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POTENTIOMETRIC AND NUCLEAR MAGNETIC RESONANCE STUDIES OF THE COMPLEXES OF ZINC(II) AND HISTIDINE-CONTAINING PEPTIDES

by

SUSAN ALEXANDRA DAIGNAULT

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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D. L. Rabenstein (Supervisor)

B. Kratochvil

P. Fedorak (External Examiner)

DATE April 30, 1985

To my family

and

tó Pierre.

ABSTRACT

The complexation of zinc(II) with glycyl-L-histidine, L-alanyl-L-histidine and glycyl-L-histidyl-L-lysine, peptides modelling the N-terminal end of the beta chain of hemoglobin, has been studied by potentiometric titration and proton magnetic resonance spectroscopy. Potentiometric titration of solutions containing peptide and Zn(II) in 1:1 and 2:1 ratios showed that in addition to the protons normally titrated from the ligand, roughly one extra proton was titrated per zinc(II) ion present. Assuming that the amide nitrogen was being deprotonated on complexation to zinc(II), a chemical model for the system was postulated and formation constants were determined from the potentiometric titration data. ^{1}H NMR experiments supported the validity of including amide-deprotonated species in the model in the appearance of resonances for kinetically stable complexes in the 6-9 ppm region of the NMR spectrum in addition to the resonances expected for the kinetically labile complexes. Fractional species distributions calculated from the formation constants determined in the potentiometric titration experiments agree well with those obtained by ^{1}H NMR, further substantiating the existence of amide-deprotonated Zn(II)-peptide species and the chemical model proposed as a whole. If the peptides used sufficiently modelled those of the N-terminal end of the beta chain of

hemoglobin, then the site of Zn(II) binding in hemoglobin may be that site; the stability of the complex due to the amide deprotonation.

An autotitrator based on the IBM PC was developed to collect the data for the Zn(II)-peptide experiments. Its performance regarding several aspects of end point and equilibrium titrations was evaluated. Diffusion of titrant from the titrant delivery tube tip was found to be negligible and the minimum volume of titrant reliably deliverable was determined to be 0.2 microliters. The precision of the determining the endpoints of titrations involving only strong acid or base was found to be about one part per thousand. It was also discovered that CO_2 absorbed in strongly basic solutions could be determined with the autotitrator for levels of carbonate in the part per thousand range. The titrator's effectiveness in collecting reliable equilibrium titration data was evaluated by determining the formation constants of complexes formed in solutions containing nickel(II) and glycine and comparison of the resulting constants with those in the literature. These experiments also help to establish the Ni(II)-glycine system as one which may be used to evaluate other titration systems such as the one used here.

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Chapter I

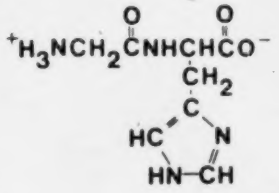
Introduction

It is well established that zinc(II) enhances the binding of oxygen by hemoglobin (1). Indeed, the use of zinc(II) has been proposed as a treatment for sickle cell anemia on the basis that it is effective in suppressing the sickling event by maintaining hemoglobin in a partially oxygenated state (1-7). Because of this potential medical importance, the nature of the interaction of zinc(II) with hemoglobin has been the subject of several investigations.

Oelschlegel (1) postulated two possible sites on hemoglobin to which zinc might bind and cause a change in hemoglobin oxygen affinity. One involved the histidine and valine residues at positions 122 and 1 (His 122 and Val 1) of the alpha chain and His 146 of the beta chain of hemoglobin, but this site was later disproven. The other was the site of 2,3-diphosphoglycerate (DPG) binding, involving residues His 143, Val 1, and lysine (Lys) 82 of the beta chain of hemoglobin (8). Early competition experiments involving zinc(II) and DPG (1) suggested there was little or no competition for the site but were inconclusive. Proton NMR studies on intact erythrocytes (9) identify a histidine residue as being a possible binding site. Several recent studies (10-14) have established the binding site as being either the amino- or carboxyl-terminal

regions of the beta chain of hemoglobin and favor the molecule's N-terminal end, possibly involving the His 2 residue.

The main objective of this thesis is to characterize the binding of zinc(II) by selected peptides having histidine in the second position from the N-terminal end as models for the proposed binding of Zn(II) by His 2 of hemoglobin. The peptides studied are glycyl-L-histidine (I), L-alanyl-L-histidine (II), and glycyl-L-histidyl-L-lysine (III).



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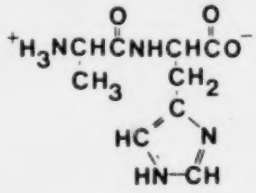

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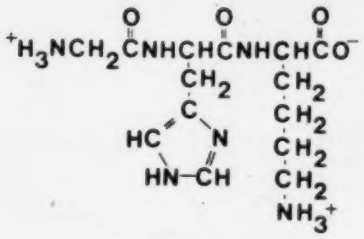


(III)

The chemical nature of the binding of transition metals to histidine-containing peptides has been studied since before 1960. The results of these studies show that many transition metals, including Cu^{2+}, Ni^{2+}, Pd^{2+}, Co^{2+}, Co^{3+}

and, to some extent, Zn^{2+}, bind to the histidine amide nitrogen as well as to amino and carboxy termina of the peptide, and that the proton on the amide nitrogen may be liberated in this process. This amide binding seems to afford the complex an added stability, as would be expected.

A. Glycyl-L-Histidine Complexes

Being a small, easily definable molecule, glycyl-L-histidine has been used in many experiments to model larger histidine-containing peptides. Thus, there have been many studies reported on the chemical behavior of this ligand, alone and in complexes, though few have involved zinc.

In one early study (15) in which zinc(II)-glyhis complexation was evaluated potentiometrically, it was found that an additional equivalent of titrant was required in titrating an equimolar solution of the metal and ligand over that expected. It was proposed that the amide proton was being displaced on complex formation as had been then and was later observed with glyhis and other metals (16-31). Ionization constants of 6.50 and 7.10 (pK_{a1} and pK_{a2}) were obtained for the complex but no association constants were determined. Also, a structure for the complex was postulated to involve coordination of the glycine amino nitrogen, the peptide nitrogen and the imidazole pyrrole

nitrogen in a tetrahedral arrangement. The carboxyl oxygen was assumed to be uninvolved.

Later, more potentiometric titration experiments were done (32) and formation constants determined for both the zinc(II)-glyhis and corresponding zinc(II)-hisgly complexes. Amide deprotonated species were found to be important in the model for the zinc(II)-glyhis complex but unimportant in the zinc(II)-hisgly complex. Another study (33) supported this. In addition to the aforementioned metal-ligand coordination sites, it was proposed that there might be coordination between the terminal carboxyl oxygen and the metal. It was proposed by these authors that there could only be one of either the terminal amino nitrogen or the imidazole pyrrole nitrogen coordinated to the metal if there was amide deprotonation, and that binuclear complexes must be included.

Most recently, the above complexes were studied by potentiometry, spectrophotometry and ^{13}C NMR spectroscopy (34). Again, amide deprotonation was found to occur only in Zn(II)-glyhis complexes and not in Zn(II)-hisgly complexes. The ^{13}C NMR experiment was done to elucidate the possibility of formation of binuclear complexes but was unsuccessful and provided information only on the very initial stages of complex formation. From the NMR data, it was deduced that an equilibrium exists between the coordination of zinc(II) by the amino and carbonyl groups and by the imidazole N_3 nitrogen of the species MLH^{2+} and ML^{+}. A structure

involving the additional coordination of the deprotonated peptide nitrogen was not described although formation constants were determined and reported. Binuclear complexes, however, were eliminated from their equilibrium model when they found that their inclusion led to a deterioration of the computer fit of their experimental data using the program SCOGS.

B. L-Alanyl-L-Histidine Complexes

The acid-base chemistry and copper and cobalt complexes of L-alanyl-L-histidine have been studied in connection with its relationship to carnosine (B-alanyl-L-histidine). The pK_a's of alahis and the formation constants of Cu^{2+}-alahis complexes were determined by Poroshin, *et al* (35). The acid-base chemistry of the histidine residue of alahis and other ligands was studied (36) by obtaining ^1H NMR titration curve data (chemical shift *vs* pH) for the C_2 and C_4 protons and determining the pK_a's from the chemical shift data. The Cu^{2+} complexes of alahis have been studied by ESR (37) and it was found that the $\rho\ x^2-y^2\ >$ ground state of the metal prevails. The conformation of the imidazole group of the histidine residue of alahis was studied (38) and found to be independent of the nature of the side chains of the residues located on either side of the histidine.

C. Glycyl-L-Histidyl-L-Lysine Complexes

Since about 1973, the biological activity of glycyl-L-histidyl-L-lysine, also referred to as glyhislys or GHL in this thesis, has been under much investigation due to its reported cell growth enhancing activity. In relation to this activity, many metal complexation studies have been done with glyhislys and while they concentrate on the copper and iron complexes, it may be appropriate to describe a little of its biological history.

Glyhislys is present in human blood plasma at a concentration of about 10^{-6} mol/dm^3(39). It was found to enhance the growth of liver cells, both in culture and from the livers of normal rats (40) and it was suggested that the mechanism of its action was through the affinity of its polar side chains for DNA (41). It has been suggested to form a ternary complex with copper(II) and albumin as does L-histidine (42,43). Copper and iron were seen to enhance the effects of GHL and it was postulated that GHL may be a copper transport factor (44). It was also shown to enhance the viability of cultured cells other than hepatocytes : fungi, lymphocytes, fibroblasts, T-strain mycoplasma, and Ascaris larvae (45-48).

It was suggested that the activity of GHL may reside in the affinity of its polar side chains for DNA (41). Studies have been done to determine the macro- and microscopic acid dissociation constants of this and related small peptides

(49) and the results suggested that the mono- and diprotonated forms, the two most abundant species at physiological pH, were the most likely to be involved in possible DNA binding.

It was found that the tripeptide was present in near equimolar concentrations with copper (40,44) and about one fifth molar with iron (40) suggesting it to be a copper- and/or iron-uptake facilitating molecule. As a consequence, virtually all studies on GHL-metal complexation have centered on its interaction with copper. The results of some of these are described here since they parallel to some extent the results obtained from the experiments for this thesis for the binding of zinc.

Early X-ray crystallography of the complex isolated at physiological pH (44) showed that the N-terminal group of glycine, the adjacent nitrogen in the first amide linkage of the peptide chain (presumed to be deprotonated although the authors do not state this), and the deprotonated nitrogen of the imidazole ring of the histidine residue formed the bonds to copper in a monomeric species. The lysine residue was uninvolved in the formation of this complex and it was postulated that the lysine might act as a receptor recognizer.

Equilibrium studies were undertaken to establish the affinity of GHL for copper in the presence and absence of albumin (43) and a complex involving a carboxy group, an imidazole, and two other nitrogen atoms coordinating in a

square planar fashion to copper was postulated. From this study, it was also suggested that the side chain amino nitrogen of the lysine residue might be involved due to the higher than expected value obtained for the formation constant for the ML species. It was suggested by the authors that the species they describe may be different from that previously described by X-ray crystallography (44). Forty five percent of the complexes they detected at pH 7.5 were ternary Cu^{2+}-GHL-albumin complexes.

EPR and electron-spin echo (ESO) studies were done to determine the structure of the GHL-Cu(II) complex in solution (50). The EPR spectrum showed the Cu^{2+} equatorially coordinated by three nitrogen atoms and ESO showed one of these to be in the histidine imidazole ring. Potentiometric titrations and spectroscopic experiments showed that at pH values elevated from physiological pH, the physiological Cu^{2+}-GHL structure was altered and that the solid state polymeric structure determined by X-ray crystallography (44) did not exist in solution but was monomeric. Again, it was suggested that the glycine and histidine residues act as metal (Cu^{2+}) chelators with the lysine residue acting as a cell surface receptor recognizer.

^{13}C and ^{1}H NMR and EPR were used to study the Cu^{2+}-GHL complex (51). EPR spectra for the copper ion showed seven lines due to nitrogen hyperfine splitting, indicating coordination of 3 nitrogen atoms to the copper. The larger

than expected formation constant (log K_f=16.44 *vs* values of 8.68 and 8.52 for similar species) suggested that another group was involved in the coordination (??). It was postulated that a carboxy oxygen atom was the additional group. The EPR spectrum showed axial symmetry indicating mononuclear Cu^{2+} complexes in an $|x^2-y^2\rangle$ ground state. The NMR data pertained only to minor species in solution and could not be used to elucidate major ones.

D. The Present Study

From the above discussion, it is clear that there is still uncertainty about how transition metals, including Zn(II), bind to histidine-containing peptides. This uncertainty is due in part to the fact that there are several potential binding sites (N-terminal amino group, peptide oxygen, deprotonated peptide nitrogen, pyrrole nitrogen, and carboxylate oxygen) and the methods used (generally potentiometry) do not provide information at the molecular level. The situation is further complicated by protonation of these sites, and thus the nature of the binding is strongly pH dependent.

In this thesis, the binding of Zn(II) by the peptides glycyl-L-histidine, L-alanyl-L-histidine and glycyl-L-histidyl-L-lysine has been studied by a combined potentiometric titration-^{1}H NMR approach. The objective of

this study is to elucidate the nature of the complexation of histidine containing peptides to the transition metal zinc. The ^{1}H NMR results showed that for all three ligands, a complex whose composition was pH independent formed over the pH range 4 to 9.5 and the complex involved binding to the imidazole ring. Proton counts from equilibrium potentiometric titrations showed that for every complex formed, one proton in addition to the number titratable from the free ligand was being titrated, possibly that residing on the peptide nitrogen coordinating to the metal. Computer analysis of this potentiometric titration data yielded likely species for a chemical model of the complexes present in the system as well as formation constants for those species. The formation constants obtained were then used to predict the relative intensities of various resonances in the ^{1}H NMR spectra and the experimental and calculated results were compared. The results of all the experiments described above are presented in Chapter IV of this thesis.

The potentiometric titration experiments were done with an automated titration system consisting of an IBM personal computer interfaced to commercially-available components for potentiometric titrations. A major part of this thesis is concerned with developing procedures with which high quality potentiometric titration data can be obtained using this system, and then evaluating the system for equivalence point and equilibrium titrations. The equivalence points of

strong acid-strong base titrations were obtained and the precision and accuracy assessed. Equilibrium data for the Ni(II)-glycine system was collected and acid dissociation constants of the ligand and formation constants of metal-ligand complexes were determined and compared with those reported in the literature. These experiments are described in Chapter III of this thesis.

Chapter II

Experimental Details

A. Chemicals

Glycine hydrochloride (Eastman), glycyl-L-histidine (Sigma), L-alanyl-L-histidine·$1H_2O$ (Sigma), glycyl-L-histidyl-L-lysine acetate (Sigma), $Zn(NO_3)_2 \cdot 6H_2O$ (Baker), $NiCl_2 \cdot 6H_2O$ (Aldrich), and $Ca(NO_3)_2 \cdot 4H_2O$ (Anachemia) were used after standardization. Potassium hydrogen phthalate (KHP) (Fisher) was used after drying for about 4 hours at 110 °Celsius. Na_2H_2EDTA (Baker 99.5%), used in metal ion standardization titrations, was first purified by recrystallization from ethanol as is described later. KNO_3 was twice recrystallized from hot water before use. Titrant KOH solutions were prepared from a DILUT-IT™ kit (Baker) while NaOH was prepared from a saturated NaOH solution. Both were used after standardization by titration of KHP.

All other chemicals (NaCl, t-butanol (Baker) etc.) were of the highest grade commercially available and were used without further purification or standardization. Doubly distilled, deionized water ($R > 5 \times 10^6$ ohm cm.) was used throughout all stages of solution preparation and in rinsing of glassware.

B. Titration Equipment Configuration

The titration system was not of a commercial design but consisted of several individually available components. The center of the system was an IBM PC equipped with a Quadboard^{TM} (320 K byte multifunction board)(52), a Tecmar PC-Mate Lab Tender^{TM} data acquisition board (53), an Intel iAPX8087 fast numeric processor chip (54), two floppy disk drives, and an Epson MX-80 printer. An Orion 701A pH meter and a Fisher 520 pH meter were modified to produce BCD output to the computer. Optical isolation of the computer and pH meters was necessary to prevent loading of the meter circuitry. An isolator was built using 12 dual MCT6 optoisolator chips and a 5 volt power supply (taken from the meter). Titrant was added with a Mettler DV11 motorized syringe buret by sending its trigger inputs a train of TTL pulses from one channel of the AM9513 timer chip on the PC-Mate Lab Tender^{TM} board.

Two titration cells were used in the experiments, one of about 15-100 mL. capacity and the other of about 5-20 mL. capacity. Each was water-jacketted and so was thermostatted at a desired temperature (±0.1 °C.) along its length when connected to a water bath. The larger titration cell was similar in design to that of Perrin and Sayce (55). The smaller titration vessel consisted of two small cells joined by a porous frit junction. This configuration allowed the same electrodes used in the larger cell to be used in small

volume experiments, thus enabling the collection of more stable pH meter readings than would have been possible with a combination electrode. The indicating electrode used was a Philips GAT130 low resistance glass (0-14 pH) electrode. The reference electrode was a Philips R44/?-5D/1 inverted glass sleeve double junction saturated calomel electrode, the liquid junction solution being of the same ionic medium as the test solutions (i.e. 1M NaCl or 0.300M KNO_3).

C. Autotitrator Software

TITRATE, written and modified in this laboratory in collaboration with Dr. A. Arnold, is the BASIC language program used to perform potentiometric titrations by controlling all components of the autotitrator. A flow chart of the program is shown in Figure 1 and a listing is included in the Appendix of this thesis.

The nature of the programming language BASIC allows the program to be interactive, enabling the user to set or change titration parameters in response to programmed prompts. If an obviously incorrect response is entered, the computer beeps and allows the user another try at a correct one, in this way attempting to be as user-friendly as possible. Also, the format for titration monitoring has been made as readable as possible. This was greatly helped by using different memory 'pages' to store parameter, meter

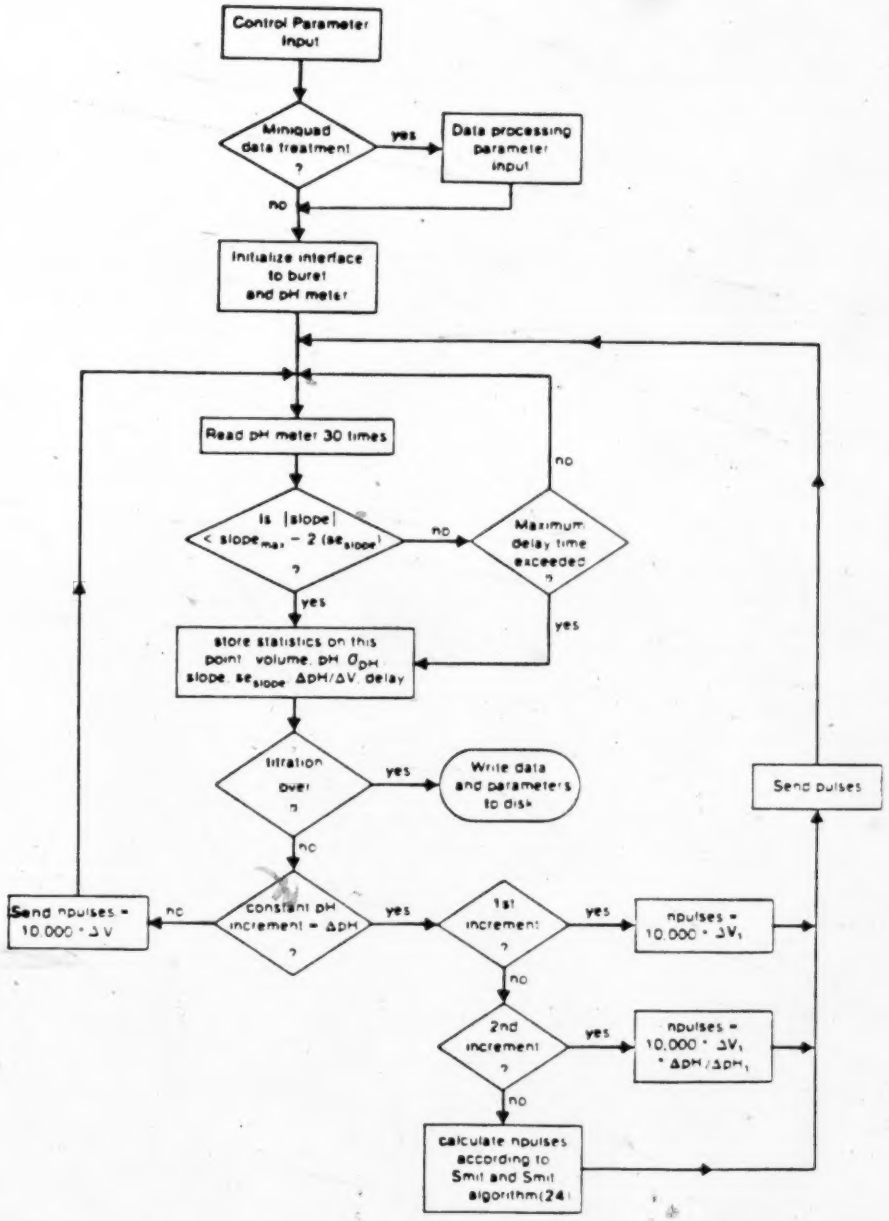


Figure 1. Flow chart for the control program TITRATE. See text for details.

test and actual titration data separately so that each was available for immediate recall by pressing one of the programmable keys of the IBM keyboard.

The first prompt given is to enter the type of pH meter used. The program works with either the Orion 701A or Fisher 520 pH meter, and that used is specified at the beginning of the program. This sets up the computer's input ports from the meter properly and specifies if the computer should expect a data ready signal (Orion) or not (Fisher). A few simple operating commands are then written to the screen. Then, the operator is given the option of having the titration data printed on paper as well as writing it to the terminal screen and floppy disk. When a hardcopy is requested, the following are immediately recorded on the Epson printer:

a) the time the program was started
b) the title of the experiment
c) headings for the data table to follow

Then, during the titration, the following is printed for each data point:

a) the number of the data point
b) the volume of titrant added up to that point
c) the pH meter reading (pH or mV)
d) the least squares slope of the collected readings
e) the slope of the titration curve between that point and the previous point $(pH_n - pH_{n-1})/(vol_n - vol_{n-1})$

f) the time the solution took to come to equilibrium

The titration parameters are then entered, or retrieved from a previously stored parameter file, as the following prompts are given: 'Is the titration in mV or pH' The answer to this question determines the position of the decimal place in the data to be collected. 'What is the end mV/pH' The answer determines the point in the titration when the program will enter a pause subroutine and wait until the operator decides to terminate or continue the titration. The operator is then asked if the titration is to be done using constant volume additions (static or equilibrium mode) or constant pH or mV increments (dynamic mode). The former is chiefly used to obtain equilibrium data in the buffered regions of a titration and so it provides the most useful data for determining equilibrium constants such as acid dissociation constants and complex formation constants. The latter is useful for endpoint determinations since a larger number of data points is collected in the titration equivalence point regions. If both types of data are required, switching between modes is simple using the parameter revision subroutine. The pH/mV or volume increment is entered next. The volume of titrant to be added to provide the required potential increment is calculated for each addition using the hyperbolic extrapolation algorithm of Smit and Smit (56). Then the criterion for determining when the test solution is at equilibrium is requested. A subroutine of the program

calculates the least squares slope and standard deviation of every 30 readings. If the standard deviation is greater than twice the standard deviation of the slope then those readings are rejected and another 30 are taken. In taking titration data, sometimes the system will seem not to come to equilibrium, for example because of a noisy pH meter or electrodes or because the stage of the titration is such that the test solution is not well buffered (i.e. equivalence point regions). To prevent inconveniently long waiting periods in these cases, a maximum waiting time is set, at which time the program takes the best readings possible from the last set collected and continues with the next titrant addition. This delay time is now entered.

The names of the files used to store these parameters and the resulting data are then entered. All parameter and data files are automatically stored on a separate disk to avoid overwriting files existing on the TITRATE disk.

The format the program will use to store the data is then determined by the answer to the prompt 'Will this data be processed with MINIQUAD81 or in another way' If MINIQUAD81 is chosen, and the MINIQUAD81 data handling parameters have been previously stored in the parameter file, they will be read and displayed on the screen for viewing while they are written to the data disk, and the collected titration data will be of the proper format to allow MINIQUAD81 processing of the data immediately after

titration termination. If these parameters have not been stored, they are then entered in answer to prompts in the same manner as for the titration parameters and will be stored with the titration parameters in the event they are needed for another titration. Details of all parameters required for MINIQUAD81 can be found in the literature (57-59), and a few of these are discussed in later sections of this chapter. These parameters cannot be changed during the titration but are easily altered afterward using the Waterloo editor.

D. pH Meter Calibration

The pH meter and electrodes were calibrated by two methods every day. First, a standard two point calibration was done using buffer solutions of pH 4.008 and 6.865, each freshly made according to NBS specifications at least every two months. This method was not completely reliable since the long term stability of the buffer solutions was questionable. The second method involved potentiometric titration of solvent solution, which contained strong acid and was prepared as described in the next section of this chapter, with strong base (NaOH or KOH) and subsequent determination of the $E^{\bullet}$ of the system by fitting the titration curve to equations 1, 7 and 8 shown following:

$$[H]_{tot} = \frac{[H]_i V_i - [OH] V_{OH}}{V_{tot}} \qquad (1)$$

where [H]tot is the total acid concentration calculated from the concentration of acid initially present and the initial solution volume ($[H]_i$ and V_i), the volume and concentration of titrant added (V_{OH} and [OH]), and the total solution volume (V_{tot}). However, to obtain an equilibrium acid concentration, which is what the electrode system measures, the autoprotolysis of water must be included in the calculations (60). Therefore, consider the following:

$$[H]_{tot} = [H]_{eq} - [OH]_{eq} \qquad (2)$$

$$K_w = [H]_{eq}[OH]_{eq} \qquad (3)$$

where K_w is the autoprotolysis constant for water and $[H]_{eq}$ and $[OH]_{eq}$ are the equilibrium concentrations of acid and base, respectively. It follows that:

$$[H]_{tot} = [H]_{eq} - K_w/[H]_{eq} \qquad (4)$$

$$[H]_{tot}[H]_{eq} = [H]_{eq}^2 - K_w \qquad (5)$$

$$0 = [H]_{eq}^2 - [H]_{tot}[H]_{eq} - K_w \qquad (6)$$

Solving for $[H]_{eq}$ gives:

$$[H]_{eq} = \frac{-H_{tot} + (H_{tot}^2 + 4 \cdot K_W)^{0.5}}{2.0} \qquad (7)$$

The simple Nernst equation was used to relate electrode potential with equilibrium acid concentration and assumed a

Nernstian electrode response.

$$E = E^{\circ} + 59.159 \cdot \log_{10}[H]_{eq} \qquad (8)$$

where E is the electrode potential and E° is the standard potential. This equation does not include terms to account for the effect of liquid junction potentials or a non-Nernstian electrode response. It was considered unnecessary to include these parameters in the calculations for the autotitrator validation experiments or for the Zn(II)-peptide experiments since they were not included in calculations done for comparable experiments reported in the literature.

The program used to solve these equations was the rigorously weighted, non-linear least squares curve-fitting program KINET which has been previously described in the literature (61). It solves for E°, K_w and acid concentration by fitting equations 1,7 and 8 to the experimental titration curve from a solvent titration. In doing so, it uses refined estimates for the above parameters with the experimental titrant volumes to calculate theoretical electrode potentials for each data point, and then compares the theoretical values with the experimental potentials. The program iterations continue until the experimental and calculated potentials agree to an arbitrary condition of 0.0001 mV. or a maximum number of iterations is performed. Since the equations involve K_w and the

concentration of acid in the test solution as well as E°, all three were typically determined simultaneously. These values were then used as constants for the processing of data collected from titrations of ligand and metal-ligand solutions of the same ionic medium.

During the latter phases of the thesis work, a program called ACBA (62), used for 'acid-base' titrations became available. This program also simultaneously fits K_w, E°, and the concentration of acid in the test solution and gives the same results as KINET (within experimental error). Typical results are presented in following chapters.

E. Preparation and Standardization of Solvent and Titrant Solutions

All solutions in this and following sections were prepared with doubly distilled, deionized water of resistivity greater than 5×10^6 ohm centimeters. The glass- and plasticware used was rinsed with it as well.

Both gravimetric and volumetric data were recorded in preparing solutions for the titrator evaluation experiments. This was because solutions used in the interlaboratory comparison study of the Ni(II)-glycine complexes (63) were prepared volumetrically, yet solution handling for the autotitrator was much more easily accomplished

gravimetrically. So that the results of this study could be compared directly with the literature results, both gravimetric and volumetric data were recorded.

All metal and ligand solutions were prepared in a 'solvent', a solution composed of a strong acid and an inert salt in water. The purpose of the salt, present in relatively high concentration, was to reduce the effect of changing solute activity coefficients and, thus, the liquid junction potentials that occur as a titration progresses in solutions of low ionic strength and the ionic concentrations change. Titrations of ligands or metal-ligand complexes that are performed on solutions of differing ionic strengths, or which have end points and buffer regions that occur at varying ionic strengths, yield rather imprecise pK_a and formation constant results (60). To illustrate, a variation in ionic strength of 10% may result in only a first decimal place precision in the logs of such constants. It was hoped that such inaccuracies could be avoided, or at least lessened, by maintaining the test and titrant solutions at a fairly high ionic strength. The purpose of the acid was to protonate the ligand and reduce the solution pH below the ligand's smallest pK_a prior to starting a titration. It also aided in keeping the metal ion in solution since both nickel and zinc hydroxides are at least partially insoluble at neutral pH (63, 64, and references therein).

HCl and NaCl were the components of the solvent for the

Ni^{2+}-glycine experiments. This solution was prepared by combining aliquots of separate HCl and NaCl solutions such that the total chloride concentration was 1.0 M. The HCl solution had been previously standardized by potentiometric titration and the NaCl, twice recrystallized from hot water before use, was assumed to be of 100% purity. For all other experiments, HNO_3 and KNO_3 were used and the solution, prepared in a similar manner, was 0.30 molal in nitrate ion. The sodium-based electrolyte, used in the Ni^{2+}-glycine experiments, was used in the present study for consistency with those experiments done in the interlaboratory study. However, in the study of Zn(II) binding by histidine-containing peptides this was replaced by the potassium-based electrolyte, KNO_3, to avoid introducing sodium error into the results.

The final acid concentration of the solvent solution was determined by potentiometric titration with standardized base using the autotitrator in dynamic mode. The dynamic mode, as described in the section on the autotitrator software, enables the collection of the maximum possible number of data points in the end point region of a titration and so ensures the best precision and, it is hoped, accuracy obtainable for the final result of a standardization titration. The change in solution pH or mV per unit titrant volume, i.e. the 'slope' of the titration curve, is calculated by TITRATE after each titrant addition. By monitoring this parameter over the course of the

titration, the end point volume is easily identified as that producing the maximum slope. The results of these standardizations are presented in following chapters of this thesis. For each solvent solution, the resulting acid concentration value was substantiated by KINET treatment of the data as described in the previous section on meter calibration.

The titrant solutions were made in the same ionic medium as were the test solutions (1.0 M $(Na^+)Cl^-$ or 0.30 molal $(K^+)NO_3^-$ to avoid complications from dilution effects. To avoid the need to include carbonate equilibria when fitting the titration data to chemical models, precautions were taken to eliminate carbonate from these solutions. The water used was first boiled and then cooled under a blanket of argon. Also, NaOH titrant was prepared by dilution of a saturated NaOH solution, which presumably is nearly void of carbonate, and the KOH titrant was prepared from a commercially available DILUT-IT™ kit (guaranteed less than 0.2% carbonate) and not from KOH pellets, which absorb H_2O and CO_2. Both were standardized by potentiometric titration of potassium hydrogen phthalate (KHP) using the autotitrator in the manner described for the standardization of acid in the solvent solutions. The KHP solutions were made from 99.9% pure KHP which had been dried for at least 4 hours in an oven containing DRIERITE at 110 °C. Results of these standardizations are also presented in

following chapters.

F. Preparation and Standardization of Ni(II) and Zn(II) Solutions

Enough $NiCl_2 \cdot 6H_2O$ was added to solvent solution to make it about 15 mM in nickel(II) and about 1 M in chloride ion. Water was added to bring the chloride ion concentration to 1.0 molar. This solution was standardized by gravimetric EDTA titration in pH 10 ammonical buffer using pyrocatechol violet and/or murexide indicator (65). Polyethylene wash bottles with hand blown small bore glass tips were used for the titrant delivery. Additions as small as 0.015 grams titrant were possible.

$Zn(NO_3)_2 \cdot 6H_2O$ was added to a solution of nitrate-incomplete solvent to give an approximate zinc concentration of 0.01 molal. This solution was standardized gravimetrically with EDTA in pH 10 ammonical buffer using Eriochrome Black T and/or pyrocatechol violet as the indicator (65). All solutions used in the studies involving zinc were prepared and standardized entirely gravimetrically to avoid the uncertainty involved in the calibration of glassware. KNO_3 was again added to make the NO_3^- concentration up to 0.300 molal and the value for the standardized zinc concentration was adjusted accordingly.

The Na_2H_2EDTA (99.5% purity) used in the metal ion standardizations was purified by recrystallization from 98% ethanol by a method reported by Vogel (66). A litre of near saturated solution of EDTA (solubility about 20 grams per 200 mL.) was prepared and 98% ethanol was added until a permanent precipitate appeared. The crystals were collected in a large Buchner funnel with suction, rinsed with a small amount of cold ethanol, and dried in an oven containing DRIERITE at about 80 °C for two days. Standardization with a primary standard $CaCO_3$ solution showed an increase in purity to 99.8% after the recrystallization.

G. Preparation and Standardization of Ligand Solutions

Glycyl-L-histidine, L-alanyl-L-histidine, and glycyl-L-histidyl-L-lysine solutions were prepared by weighing the solid into measured amounts of solvent. Solutions were standardized by potentiometric titration with subsequent data work-up using the non-linear least squares curve-fitting programs MINIQUAD81 (59) (a 1981 version of MINIQUAD (57,58)) or ACBA (62), though ACBA was not available for use until very late in this research.

When MINIQUAD81 was used to calculate the ligand concentration from pH titration data, either the titration program 'TITRATE' was used to put the data in the proper

format for MINIQUAD81 as it was being collected, or a data file of the proper format was set up manually after completion of the titration. Included in a data file for MINIQUAD81 are a title, the titration temperature, the E° of the system, the pK_a's and stoichiometric coefficients of each species in solution, the number of mmoles of each component in the solution to be titrated and titrant, the initial test solution volume, and the potentiometric titration data (in pairs of titrant volume and corresponding pH or mV reading).

To run MINIQUAD81, K_w and, initially, E° were kept constant at the values determined from ACBA or KINET treatment of solvent titration data as described in the pH meter calibration section of this chapter. It is possible for MINIQUAD81 to determine values for K_w and E° but it was found that refining too many parameters at once gave unreliable results.

It was found that the stability constants determined using MINIQUAD81 were highly sensitive to the values of the parameters kept constant, such as ligand concentration, E°, K_w, etc. To determine the actual ligand concentration, it was necessary to run the program a number of times using different estimates for the number of mmoles of ligand and monitor the fit of the results to the data as indicated by the sum of squares of the residuals value, which is calculated from the sum of the squares of the differences

between each calculated and experimental titration data point. The number of mmoles present was taken to be the value that resulted in the smallest sum of squares value. Figure 2 shows the relationship between the sum of squares parameter and the mmoles of ligand used in the calculation. A better fit of the calculated and experimental titration curves results when the value for the mmoles of ligand approaches an optimum. Seven titrations of glyHCl gave a mean concentration of 0.0229(5) molar. Similarily, results for the other ligands were 0.01058(3) molal glyhis, 0.0244(1) molal alahis (8 replicates each) and 0.01360(5) molal glyhislys (4 replicates), where the values in brackets refer to the standard deviation in the result.

ACBA needed only be run once to obtain values for the concentration of ligand, K_w and E° simultaneously. Data files for ACBA were set up with the same information as for MINIQUAD81 but in a different format. The program has the capability of refining K_w and E° at the same time it refines pK_a's and acid and ligand concentrations, seemingly with better reliability and certainly with greater ease than with MINIQUAD81. The values calculated for E°, K_w, and acid concentration agreed well with those obtained by solvent titration, as described in section D of this chapter, making the titration of the solvent seem unnecessary. However, as with most curve-fitting programs, the more parameters there are to be refined the less reliable are the results. Thus, the E° and K_w values used in the ACBA calculations were

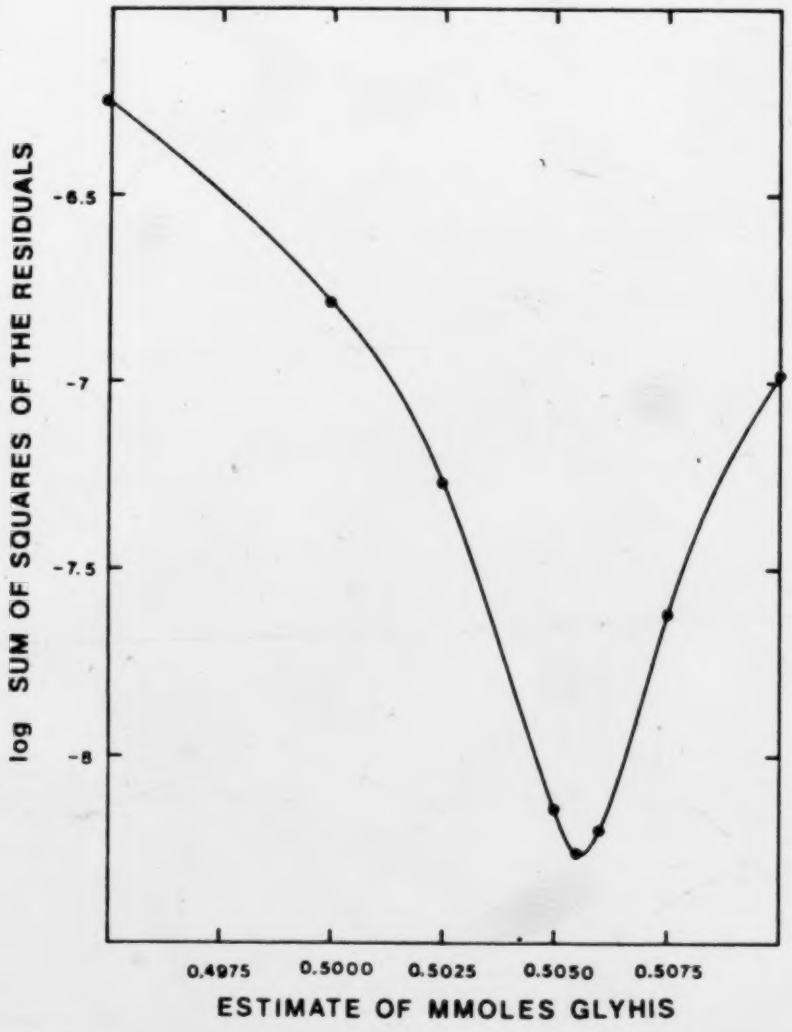


Figure 2. Dependence of the value of MINIQUAD81's sum of squares of the residuals parameter on the value used for the mmoles of ligand.

determined by titration of solvent, and the ligand pK_a's and concentrations were obtained from the ACBA calculations.

H. Solutions for the ^1H NMR Experiment

Solutions used in these experiments were prepared using the autotitrator, or the Mettler DV11 buret alone, to deliver aliquots of base (NaOH or KOH) into the test solution containing ligand or a metal/ligand mixture. The base was of the same ionic medium as the test solution to keep the ionic strength of the test solutions constant at a value similar to that used in the potentiometric experiments.

Ligand solutions were prepared by weighing solid ligand into a flask with a measured amount of solvent, taking into account the purity determined for the ligand in the separate potentiometric titration experiments described in the previous section. An aliquot of this solution was transferred to the titration cell. If the test solution was to contain metal ion, an aliquot of standardized metal ion solution was then measured into the cell. The initial pH of the solution was adjusted, if necessary, to between 2 and 4 with solvent. Tertiary butyl alcohol (TBA) was added as an internal chemical shift reference at a concentration of about 5×10^{-4} molar. The final concentrations of ligand and metal in the test solution at the start of the titration

were in the range of 2-15 mmolar with ligand to metal ratios ranging between 1:1 and 5:1.

Solutions were bubbled with argon for about 5 minutes before starting the titration and were continuously bathed in argon during the titration to minimize absorption of CO_2 which would alter solution pH. Typically, ten to twenty five 0.5 mL samples were taken from the titration cell during the titration and put in NMR tubes. The pH range covered was typically from pH 2 or 4 to about pH 11, with samples being taken at regular pH intervals. No attempt was made to correct pH values obtained for these solutions, which contained 1% D_2O, for deuterium isotope effects. Proton NMR spectra were measured within half a day of sample preparation.

I. ^1H NMR Measurements

Proton NMR spectra were obtained using a Bruker WM 360 high resolution spectrometer equipped with an Aspect 2000 computer. The probe temperature was kept constant at 25 ±1°C.

A pulse width of 1-3 micro seconds was used. To achieve adequate signal to noise ratio, between 40 and 120 free induction decays were averaged. Chemical shifts were measured relative to the pH insensitive t-butyl resonance of

t-butanol (TBA, chemical shift 1.2365 ppm). The solutions contained 1% v/v D_2O to provide a deuterium lock signal for the spectrometer. To avoid the dynamic range problem encountered when measuring spectra of dilute solutes in water, the H_2O resonance was reduced in intensity with a selective saturation pulse prior to applying the nonselective observation pulse.

Peak areas were obtained by both using the integration software of the instrument and by cutting and weighing the peaks from the printed spectra.

J. Ligand Acid Dissociaton Constant Determinations

Solutions of ligand in solvent at an initial pH of about 2 were titrated with standardized base using the autotitrator. The data were processed in one of two ways: (a) using the program MINIQUAD81 (59) or (b) ACBA (62). The same results were obtainable with either program (within experimental error) although it was easier to use ACBA since it is a one-run program, as has been discussed earlier.

With MINIQUAD81, it was necessary to run the program several times to obtain the final results. First, the number of mmoles of ligand in the titrated solution was determined as described in section G of this chapter. In the case of glyhislys, this was done twice - once to obtain

the mmoles of acetate present and once to obtain the mmoles of glyhislys. If the titrations were done in mV mode, MINIQUAD81 was run again, this time varying a parameter called PHCAL which attempts to correct for an inaccurate E° value. Typically, this affected the E° value by less than a few millivolts, but did affect the proton dissociation constants somewhat and resulted in better fits to the data. When the mmoles of ligand and E° were optimized, the values for the overall dissociation constants were calculated.

MINIQUAD81 only calculates overall association constants, called betas, which are described by the general relationships shown below:

$$aL + bH \rightleftharpoons L^{a}H^{b} \tag{9}$$

$$\beta_{ab} = \frac{[L_aH_b]}{[L]^a[H]^b} \tag{10}$$

where L and H represent the ligand and protons, respectively, and a and b represent their stoichiometric coefficients. Thus, for example, for a triprotic ligand:

$$\beta_1 = \frac{[HA]}{[H][A]} \tag{11}$$

$$\beta_2 = \frac{[H_2A]}{[H]^2[A]} \tag{12}$$

$$\beta_3 = \frac{[H_3A]}{[H]^3[A]} \tag{13}$$

Equations 14 to 26 are employed to convert these constants to conventional acid dissociation constants.

$$K_{a1} = \frac{[H][H_2A]}{[H_3A]} \quad (14) \qquad K_{f3} = \frac{[H_3A]}{[H][H_2A]} \quad (17)$$

$$K_{a2} = \frac{[H][HA]}{[H_2A]} \quad (15) \qquad K_{f2} = \frac{[H_2A]}{[H][HA]} \quad (18)$$

$$K_{a3} = \frac{[H][A]}{[HA]} \quad (16) \qquad K_{f1} = \frac{[HA]}{[H][HA]} \quad (19)$$

$$-\log K_a = \log K_f \quad (20)$$

$$\log\beta_1 = \log K_{f1} = -\log K_{a3} \quad (21)$$

$$\log\beta_2 = \log K_{f1} + \log K_{f2} = -\log K_{a3} - \log K_{a2} \quad (22)$$

$$\log\beta_3 = \log K_{f1} + \log K_{f2} + \log K_{f3} = -\log K_{a1} - \log K_{a2} - \log K_{a3} \quad (23)$$

where β represents the overall association constant, K_f the stepwise association constant, and K_a the stepwise dissociation constant. From the above, it follows that the ligand proton association constants can be simply converted from MINIQUAD81 beta values to conventional pK_a's by equations 24 to 26.

$$pK_{a1} = \log\beta_3 - \log\beta_2 \quad (24)$$

$$pK_{a2} = \log\beta_2 - \log\beta_1 \quad (25)$$

$$pK_{a3} = \log\beta_1 \quad (26)$$

The mean and standard deviation of pK_a values determined from the treatment of data from several titrations were calculated using equations 27 and 28:

$$\text{Weighted mean} = \frac{\sum(x/sd^2)}{\sum(1/sd^2)} \qquad (27)$$

$$\text{Weighted standard deviation} = 1/\sqrt{\frac{\sum(sd^2)}{n-1}} \qquad (28)$$

where x represents each individual result, sd is its standard deviation, and n is the number of results used in the calculation (67). The Q test (68) was used to determine if outlying pK_a values should be included in the results.

In calculating the weighted mean for a set of log values, the inverse log was first calculated and used for x. The nature of the weighting is such that the values with larger error associated with them, as indicated by their standard deviations, influence the final result to a much lesser extent than those with relatively little error. This increases the confidence in the final result by weighting more heavily the contribution of the values of which there is more certainty and also by lessening the magnitude of the standard deviation associated with the final result. In the case of the Ni(II)-glycine experiments, simple means and standard deviations were also calculated for the pK_a's to allow direct comparison of the experimental values with those of the literature.

K. Potentiometric Determination of Metal-Ligand Formation Constants

At the completion of a ligand titration, acidified metal ion solution was measured into the titrated ligand solution. The metal/ligand solution was then titrated with base using the autotitrator. Data collected from these experiments was processed using the program MINIQUAD81 (59) to determine metal-ligand formation constants in much the same way as was done in calculating ligand pK_a's. The solution K_w, ligand pK_a's, and, initially, E^c which were determined from solvent titrations were kept constant in these calculations, as was the number of mmoles of ligand determined in the previous titration. In the calculation of the Zn-ligand formation constants, it was found that inclusion of the species $Zn(OH)^+$ and $Zn(OH)_2$ in the model was necessary for a good fit. Formation constants for $Zn(OH)^+$ and $Zn(OH)_2$ were obtained from separate titrations of zinc solution and are reported in Chapter IV.

Since the computer program MINIQUAD81 was used extensively in this thesis research to calculate formation constants, the procedures for using the program and some of its capabilities will be described. First, an equilibrium model is proposed for the system under study. Stoichiometric coefficients of the components (metal, ligand and protons) of all possible species and estimates for the formation constants of the various species represent the

model system in the program as described in the Appendix. These are used by the program to set up mass balance equations for each element. The actual experimental condition values, i.e. the initial solution temperature and volume, system F°, and the concentrations of all the components making up the species in the model, are used along with the above mass balance equations to simulate a titration curve. This titration curve is then compared point by point with that obtained experimentally under the given conditions and, on the basis of the results of the comparison, the formation constants are refined by means of a non-linear least squares algorithm. This process is repeated until an arbitrary criterion of fitness is achieved or a maximum number of iterations is performed.

Included in the algorithm is the capability to recognize when successive iterations for the calculation of the constant for a particular species proposed in the model is not converging to a single value. Subsequently, that species is rejected from the model while the constants for the remaining species are further refined.

Relative errors in the final constant values are calculated along with several different estimates of the overall fit of the experimental data to these results for each data set. The only one of these that will be discussed in this thesis is the sum of squares of the residuals parameter, or simply the 'sum of squares', which is the sum of the squares of the differences between the experimental

data points and those calculated using the resulting constants. In other words, it gives a measure of the fit of a titration curve calculated using MINIQUAD81 constants to that obtained experimentally. Although determining the degree of the fit by the value of the sum of squares parameter is somewhat arbitrary, it is generally observed that a calculated titration curve will pass through most all experimental titration points with no observable deviations from the experimental curve in any specific region, such as at an end point, in a buffer region or in regions of either high or low pH, when the sum of squares value is less than 10^{-7}.

The program may be run such that several equilibrium models are successively fitted to a set of data, i.e. different combinations of species may be included in each trial run. This is referred to as the 'model select' mode in this thesis. By monitoring the sum of squares parameter and the frequency of rejected species, a theoretically 'best' chemical model for the system can be surmised. Care must be taken to include only species having some chemical validity since the program will initially accept any species, whether it makes chemical sense or not. Similarily, if a species is rejected by MINIQUAD81 it does not necessarily mean that it is totally invalid to the system since the equations for species present at low levels are difficult to solve. However, those species repeatedly rejected by the program were generally rejected by this

author unless there was a very sound chemical reason to include them. Similarily, those with highly irreproducible values were also held highly suspect. Suspect species were confimed or rejected by running the data with a species distribution calculating program COMIX, described in the next section. Generally, a species with an abundance of less than 5 percent was rejected from the model.

For some of the less abundant species which seemed chemically reasonable, it is difficult to get reliable constants. By running the program several times and successively keeping the estimates of the more abundant species at values to which they had become constant, it was possible to enhance the reliability of the constants for these minor species.

Because of the variability in the values of the formation constants due to various of the above factors, weighted means and standard deviations were calculated using equations 27 and 28 for all the constants as shown in the previous section of this chapter. Simple means and standard deviations were also calculated for the Ni(II)-glycine system to permit a direct comparison of the results of this study with those of others which did not use such weighting in their calculations.

L. The Compatibility of Potentiometric and NMR Results

Many laboratories study complex equilibria by potentiometric titration methods similar to those used here. The validity of the results of such experiments can be greatly enhanced by comparison with the results of separate confirmation experiments involving the use of a different experimental method. In this research, the potentiometric results have been compared with the results obtained by ^1H NMR.

After determining formation constants from a particular set of titration data by fitting it to a model with MINIQUAD81, the program COMIX (69) was run with those formation constants and the same metal, ligand and proton concentrations to calculate theoretical distributions of the various species. The quantitative results of the ^1H NMR experiments, i.e. the relative peak areas from the imidazole region of the spectra, were compared with the theoretical species distributions obtained above. Agreement was considered to be good if the differences between the concentrations predicted by COMIX and those observed by NMR were within experimental error, which was considered to be about 10 percent. Obtaining such agreement was taken to indicate that the species belongs in the proposed model and that the formation constants obtained for it are valid.

CHAPTER III

Evaluation of the IBM PC Autotitrator

A. Introduction

The results of an exhaustive testing of the IBM PC controlled autotitrator described in Chapter II are presented here. Several facets of the system were examined and the limitations in its ability to perform endpoint and equilibrium titrations was ascertained.

The minimum volume of titrant deliverable was determined, as was the presence or absence of diffusion from the delivery tube tip. Tests were done to see how accurately and reproducibly endpoints of strong acid/strong base titrations, done in either the large (20-100 mL) or the small (5-25 mL) titration cell, could be obtained. The determination of small amounts of a component in the presence of larger amounts of another (eg. 1 ppt. carbonate in 0.2 molar KOH) was also evaluated.

The reliability of using the titrator for collection of equilibrium data was investigated by determining the formation constants of the nickel(II)-glycine complexes and comparing the results with those in the literature (63). The nickel(II)-glycine system is chemically well behaved, the the reactions are not greatly endo- or exothermic, and there

no precipitation before pH 9. The system is sufficiently complex that there are several species present simultaneously in solution but it is not so complex that unusual measurement or calculation procedures are needed. Formation constants for nickel(II)-glycine complexes have been measured by several laboratories and an international interlaboratory determination of the formation constants (63) compares the values obtained using different experimental and computational methods. Results from the interlaboratory comparison have been treated statistically (70,71), providing what would seem to be an excellent chemical system for the evaluation of an equilibrium titrator.

B. Results

1. Dynamic Titrations

Dynamic titrations were defined in Chapter II as being those in which each titrant volume addition was calculated to allow the data points to be obtained at constant pH intervals. This method enables the collection of many data points in the regions of the equivalence points and so is useful in determining the concentration of titratable solution components.

a. Diffusion from the Titrant Delivery Tip

Solvent solution, defined in Chapter II, was titrated in dynamic mode with the autotitrator using 0.875 M NaOH to within 2 pH units of the equivalence point (pH 7) and the titration then suspended to monitor changes in the pH meter readings. Meter drift would be expected if titrant was diffusing from the delivery tip.

In the first experiment, the tip was removed on suspension of the titration. In 3 runs the meter readings drifted an average of about 0.025 pH units during the first 2 minutes after removing the tip and then remained constant, where constancy was defined arbitrarily by a drift in pH of less than 0.001 per minute. In the second experiment, the delivery tip remained in the test solution after suspending the titration and the meter readings were found to drift about 0.03 pH units over the initial 2 minute time interval before becoming constant. Thus, diffusion from the titrant delivery tube tip was taken to be negligible.

b. Minimum Titrant Volume

In dynamic titrations, the titrant volume to be added is determined by application of an hyperbolic algorithm (56) to the two previous data points. In the equivalence point region of a strong acid/strong base titration, such calculations can result in theoretical titrant additions of

volumes less than that possible with the DV11 motorized syringe buret, where the 1 mL buret has a documented minimum volume addition of 0.0001 mL. To determine if the minimum volume which can be added is actually 0.0001 mL, solvent was titrated and the endpoint region was monitored as titrant was added. A gradual increase then decrease in the TITRATE-calculated slope parameter, which is the change in pH or mV reading per change in titrant volume as described in Chapter II, would be expected if the volume additions were being made properly.

Minimum volume additions of 0.0002 mL produced such a response while those of 0.0001 mL did not. Additions of 0.0001 mL titrant in the equivalence point region of the titration produced what will be referred to as an oscillating slope. One titrant addition would produce a great change in the pH or mV reading and, therefore, in the calculated slope. The next titrant addition might cause a much smaller change in the meter reading and slope. The trend would continue through the equivalence point.

It is possible that there was an electronic problem. Since schematics were unavailable for the buret, it could only be postulated that its mechanism might be such that both a rising and a falling pulse needed to be applied to its trigger to move the piston and expel titrant. This explanation was ruled out, however, since the computer always applies a rising pulse to the buret as its first pulse. If the above were true, such applications of single

pulses would not be expected to move the buret piston at all, and the piston was seen to move on multiple single pulse applications.

It was noted that the buret made a clicking noise when titrant was being added, and examination revealed a small space between the piston and its driver. It might be that, when a pulse is applied, the driver moves but the piston does not move enough, or perhaps it moves too much. On switching buret drive boxes, the clicking noise and the oscillating slopes both decreased, but did not disappear. It was concluded that the oscillating slopes were due to a hardware defect that could not be eliminated.

In any event, for all dynamic titrations a minimum titrant volume addition of 0.0002 mL was added when the volume calculated by the hyperbolic algorithm was less than 0.0002 mL.

c. Accuracy and Precision of Endpoint Titrations

The most important aspects of endpoint titrations are their accuracy and precision. To determine the accuracy and precision of the endpoint detection, the same solvent titration experiment described in the two preceeding sections was performed. The accuracy of the autotitrator's endpoint detection was assessed by comparing the endpoint

obtained by taking the point having the maximum calculated slope, which will be called a manual calculation or a derivative result, and that obtained by treating the data with the program KINET (61). This is not to imply that the evaluation of the data by KINET is definitive in obtaining true values for the concentrations of solution components. Rather, the correlation of the derivative and KINET results, in conjunction with obtaining good precision as calculated by standard statistical methods, was thought to merely indicate a level of accuracy and not to define an absolute accuracy for the values.

In the endpoint region of the titrations, a minimum volume increment of 0.0002 mL was made, which limits the accuracy and precision of the results to 0.2 ppt. if the endpoint occurs at a titrant volume of about 1 mL.

For titrations done in the large cell, 5 replicate titrations of about 35 grams of solvent, each requiring about 0.5 mL. titrant, resulted in an average acid concentration of 0.009431 molal with an average deviation of 0.000007(0.7 ppt.). In another experiment, done in the small titration cell and using different titrant and solvent solutions, 4 replicate titrations of about 6 grams of solvent, each requiring about 1.2 mL of titrant, gave an average acid concentration of 0.04475 molal with an average deviation of 0.00005 (1.1 ppt.).

The same data was run with the program KINET. The result obtained for the titrations in the large cell was

0.009422 molal H^+ with an average deviation of 0.8 ppt. while the result obtained from titrations in the small cell was 0.04477 molal H^+ with an average deviation of 1.0 ppt. A comparison of the derivative- and computer-calculated results for the data collected with the large cell is given in Table 1.

On the basis of these results, it was concluded that the endpoints of strong acid/strong base titrations done on the IBM PC controlled autotitrator, whether determined by monitoring the titration curve slope or by computer analysis of the data, can be determined reliably to 1 ppt. or better.

d. Low Level Components in Multicomponent Solutions

Sometimes, relatively small concentrations of contaminants will have a large effect on the behavior of a chemical system. For example, basic solutions will absorb CO_2 from the atmosphere and in doing equilibrium studies it might be necessary to include carbonate protonation equilibria in the chemical model. To determine if low levels of carbonate could be detected in KOH titrant, solvent solution was titrated with a dilute solution of freshly prepared KOH stock solution containing about 1 ppt. carbonate. In all stages of solution preparation, only doubly distilled, deionized boiled and deaerated water was

Table 1.

Solvent Free Acid Standardization Using KINET and by Derivative Calculation[a,b]

DATE/FILE	KINET RESULTS	MANUAL RESULTS[c]
MAY02002	9.408(3)	9.415(7)
MAY02004	9.41(4)	9.437(7)
MAY02005	9.423(4)	9.429(7)
MAY02006	9.41(4)	9.439(7)
APR29004	9.431(2)	9.433(7)
AVERAGES	9.422(7)	9.431(7)

[a]Concentrations are given in units of 10^{-6} moles strong acid per gram solution.

[b]Numbers in parentheses represent the uncertainty in the last digit as measured by the linear estimate of the standard deviation as calculated by the program KINET (KINET results) and by assuming an error of ±0.0002 mL in the endpoint volume and ±0.002 grams in solution weight and using these values in standard error analysis of the results (MANUAL results).

[c]MANUAL results are those determined from the titration curve derivative calculations done by TITRATE during the titration.

used and the KOH stock solution was stored in an air tight polyethylene bottle. Later, the reverse titration experiment, that of base with solvent, was also done using a fresh KOH solution made from dilution of the initial KOH stock solution.

First, solvent solution (0.04564 M acid with a standard deviation of 0.00008 M) was titrated with carbonate-containing KOH titrant. The high level of performance of the titration system is evident from the observation of 2 maxima in the derivative plot (Figure 3), separated by only 1.3 microliters. The two end points result from the presence of carbonate in the titrant and it can be surmised that the base strength at the first end point is $[OH^-] + 2[CO_3^{2-}]$ while at the second it is $[OH^-] + [CO_3^{2-}]$. Typically, in 4 replicate titrations, 5 additions of the minimum possible amount of titrant (0.2 microliters) were made between the two end points, and gave reproducible derivative titration curves. The end point volumes were identical to those obtained from Gran's plots (72). The average titrant carbonate concentration was found to be 0.89 ppt. with an average deviation of 0.09 ppt. Derivative curves for simulated titrations of strong acid with strong base containing 1 ppt. carbonate, one of which is shown in Figure 4, are identical in shape to the experimental derivative curves. The carbonate concentration calculated by these simulations was 0.85 ppt. with an average deviation of 0.09 ppt. compared with the experimental derivative curve

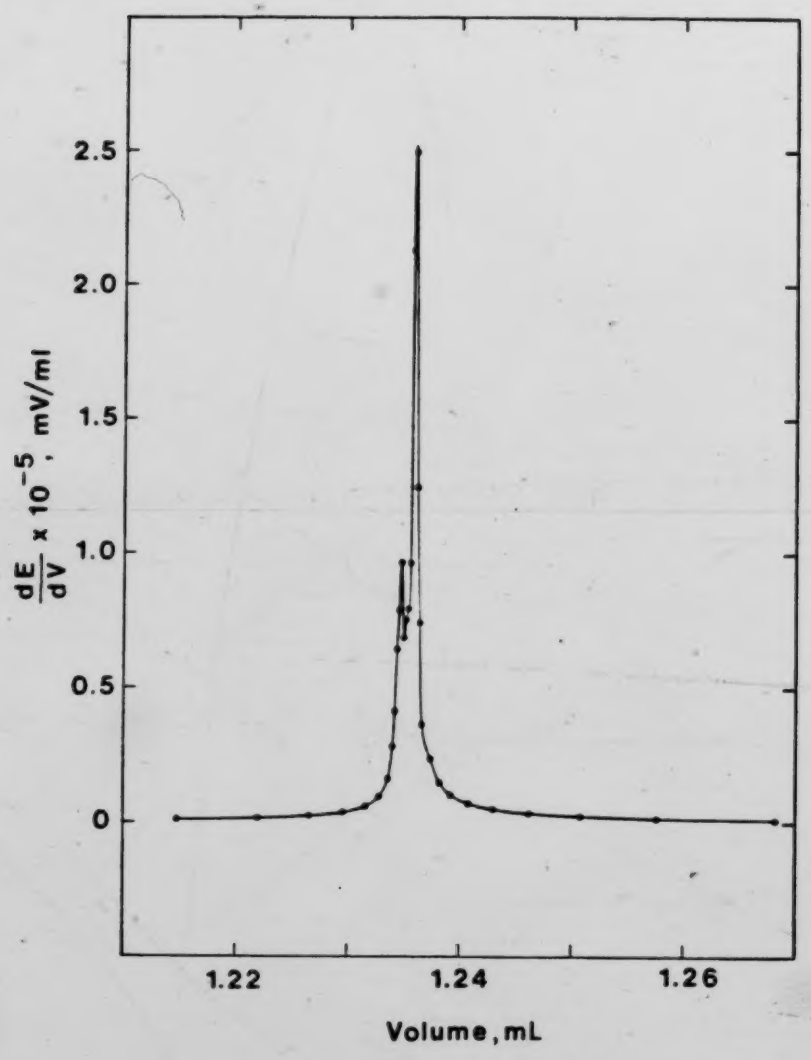


Figure 3. Derivative curve in the equivalence point region of a titration of 0.045 M HNO_3 with 0.2 M KOH in 0.3 M KNO_3.

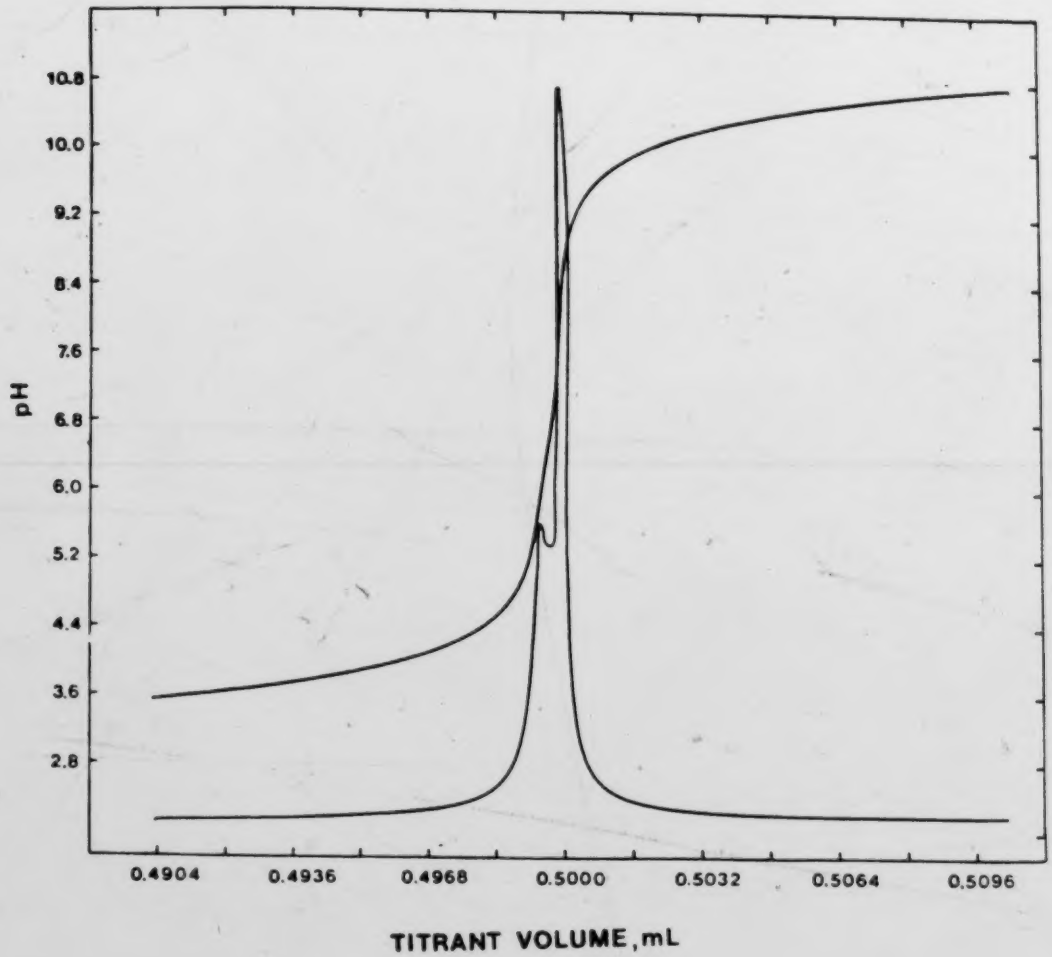


Figure 4. Simulated titration and derivative curves for the titration of strong acid with strong base containing 1 ppt. carbonate.

calculated value of 0.39 ppt. with an average deviation of 0.00 ppt.

The reverse experiment was also performed, using the same solvent solution and a diluted aliquot of the stock titrant solution from which the titrant in the first experiment was prepared. Again, derivative plots of the titration curves showed two maxima as had been observed in the previous experiment. In this instance, the first end point represents the titration of OH^- to H_2O plus the titration of CO_3^{2-} to HCO_3^- while the second end point results from the titration of HCO_3^- to H_2CO_3. Five replicate titrations gave a carbonate concentration for the base of 1.6 ppt. with an average deviation of 0.1 ppt. and, again, the experimental and simulated titration curves were identical in shape. These results suggest that it is possible to quantitate less than 2 ppt. carbonate in strong base titrant with the autotitrator by titrating it with strong acid or even by simply using it to titrate a strong acid solution.

It was noted that the actual values for the concentration of CO_3^{2-} from the two experiments did not match. There are two possible reasons for the observation, one being that the KOH had simply absorbed more carbonate in the month between the first and second experiment. For a second explanation, consider the following equilibria:

$$H_2CO_3 \rightleftharpoons CO_2 + H_2O \qquad (29)$$

$$H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \qquad (30)$$

$$HCO_3^- \rightleftharpoons CO_3^{2-} + H^+ \qquad (31)$$

In acidic solutions, carbonate is converted to carbon dioxide and will escape into the atmosphere. When base containing carbonate enters the acidic environment of the test solution, acid neutralizes the base and, depending on the pH, converts some of the carbonate to carbon dioxide which then may escape the solution. In the opposite titration, the acid entering the basic titrate neutralizes some of the base much more quickly than it can react with carbonate and so little of the carbonate is converted to carbon dioxide. This may be why the first experiment gave lower results for the concentration of carbonate than does the second.

2. Equilibrium Titrations

Equilibrium titrations were defined in Chapter II to be those in which the volume of titrant added to the test solution for each data point is constant, thus enabling the collection of many data points in the buffer regions of these titrations. In this way, the most reliable equilibrium information, i.e. proton dissociation constants, complex formation constants, etc., can be obtained from the

titration data.

a. The Acid Dissociation Constants of Glycine

The acid dissociation constants of glycine were determined by titration of solutions of the hydrochloride salt of glycine with NaOH. The hydrochloride salt of glycine dissociates in solution to form protonated glycine and chloride ion as shown below.

$$^{+}H_3NCH_2COOHCl^{-} \rightleftharpoons {}^{+}H_3NCH_2COOH + Cl^{-} \qquad (31)$$

The contribution of the Cl^{-} from this reaction to the total ionic strength of the solution was taken into account when preparing the solutions.

The glycine molecule, when fully protonated, is a dibasic acid which dissociates according to equations 32 and 33.

$$^{+}H_3NCH_2COOH \rightleftharpoons {}^{+}H_3NCH_2COO^{-} + H^{+} \qquad (32)$$

$$^{+}H_3NCH_2COO^{-} \rightleftharpoons H_2NCH_2COO^{-} + H^{+} \qquad (33)$$

The acid dissociation constants which describe these reactions are defined as:

$$K_{a1} = \frac{[H^{+}][^{+}H_3NCH_2COO^{-}]}{[^{+}H_3NCH_2COOH]} \qquad (34)$$

$$K_{a2} = \frac{[H^+][H_2NCH_2COO^-]}{[^+H_3NCH_2COO^-]} = \frac{[H^+]^2}{K_{a1}[^+H_3NCH_2COOH]} \qquad (35)$$

K_{a1} and K_{a2} were determined by potentiometric titration of acidified solutions of glycine hydrochloride, as described in Chapter II of this thesis. The constants were obtained from the titration data with the program MINIQUAD81. All solutions were titrated at a temperature of 25 °C and had an ionic strength of 1.0 M $(Na^+)Cl^-$, as described in Chapter II. The concentration of glycine in the titrations was about 0.02 M and was standardized as will be described next.

The glycine concentration was determined by dynamic potentiometric titration and monitoring the slope parameter calculated by TITRATE after each titrant addition to determine the equivalence points. The first equivalence point was due to the titration of free acid in solution. The second was due to the titration of two protons from the ligand. Using the difference in titrant volume between the two equivalence points, a concentration of 0.02286 mmoles per gram of solution with an average deviation of 0.00045 mmoles per gram solution (2 %) was obtained. The relatively low precision was due to the relatively small change in pH at the equivalence points.

The K_a's were then determined with MINIQUAD81, using values for the F°, K_w, and mmoles free acid determined from the solvent standardization experiments, as well as the

experimental data from the ligand standardization experiments. The results are presented in Table 2 and are compared with those of an interlaboratory study (63). The nature of the equations used to weight the individual data (equations 27 and 28) is such that the values with larger error associated with them, as indicated by their standard deviations, influence the final result to a much lesser extent than those with relatively little error. This increases the confidence in the final result by weighing more heavily the contribution of the values of which there is more certainty and also by lessening the magnitude of the standard deviation associated with the final result. This explains why some of the final results in Table 2 and in following tables have associated with them smaller standard deviations than would be expected from simple, non-weighted calculations.

Figure 5 shows typical experimental titration data. The solid curves through the experimental points are theoretical curves calculated using the constants reported in Table 2.

b. The Formation Constants of Nickel(II)-Glycine Complexes

The nickel(II)-glycine system has been extensively studied. An international interlaboratory study of the

Table 2.
pK_w, and Glycine Concentration and pK_a Determinations
Using MINIQUAD81[a,b]

DATE/FILE	Conc. glyHCl[c]	pK_w	pK_{a1}	pK_{a2}
MAR09001	0.022774	13.905(1)[d]	2.468(4)	9.779(1)
MAR10004	0.023208	13.905(4)	2.446(6)	9.773(3)
MAR21003	0.023761	13.696(9)	2.32(7)	9.53(3)
MAR22003	0.023006	13.679(5)	2.500(9)	9.680(6)
APR22007	0.023281	13.723(2)	2.50(4)	9.617(7)
MAY03001	0.022330	13.643(2)	2.43(2)	9.553(8)
MAY03002	0.021940	13.643(8)	2.43(5)	9.568(2)
AVERAGE[e]	0.0229(5)	13.74(9)	2.44(12)	9.64(9)
WT'D.AVG.[f]	------	13.83(1)	2.48(1)	9.74(1)
LITERATURE[g]	------	13.69(2)	2.43(3)	9.65(1)

[a][NaOH] = 0.8754 M
[b]$[HCl]_{solvent}$ = 0.009431 molal
[c]Concentration in units of molal.
[d]Values in brackets are the standard deviations in the least significant digits of each value.
[e]Simple mean and standard deviation not considering the standard deviation in each individual result.
[f]From equations 27 and 28.
[g]Reference 63.

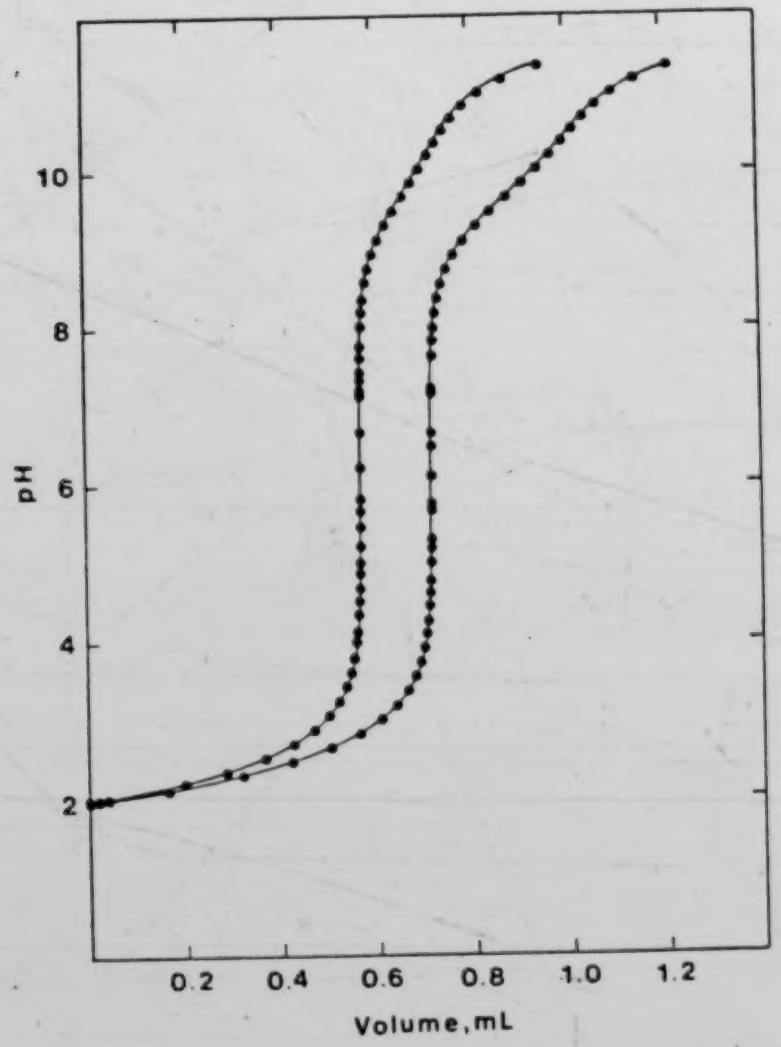


Figure 5. Typical experimental (●) titration curves and corresponding theoretical (—) titration curves calculated using the pK_a's shown in Table 2 for solutions containing 0.018 and 0.022 M glycine. Every second data point is plotted.

complexes was done (63) and the formation constants of its nickel complexes, as defined by the following equilibria, were determined.

$$Ni + L \rightleftharpoons NiL \quad (36)$$

$$Ni + 2L \rightleftharpoons NiL_2 \quad (37)$$

$$Ni + 3L \rightleftharpoons NiL_3 \quad (38)$$

where L represents the totally deprotonated glycine molecule and Ni represents Ni^{2+}. The corresponding overall concentration formation constants, designated by βs, are defined as:

$$\beta_{110} = \frac{[NiL]}{[Ni][L]} \quad (39)$$

$$\beta_{120} = \frac{[NiL_2]}{[Ni][L]^2} \quad (40)$$

$$\beta_{130} = \frac{[NiL_3]}{[Ni][L]^3} \quad (41)$$

Potentiometric titrations to determine the formation constants were performed as described in chapter II of this thesis over a period of six weeks. Solutions were maintained at a temperature of 25 °C and were of 1.0 M ionic strength, predominantly NaCl. Standardized nickel, ligand, and solvent solutions were measured into the large titration cell to give solutions of between 0.001 and 0.010 molar

glycine concentration and between 0.001 and 0.007 molar nickel concentration having glycine:nickel ratios of between 5:1 and 1:1 and a total initial volume of about 40 mL. For consistency with previous studies, only the data collected between pH 2 and 9.5 was used in the formation constant determinations.

If nickel precipitated from solution, the titration was terminated. Precipitation could be detected before the precipitate was visible by longer than normal equilibration times and drifting pH meter readings.

The data was processed with the program MINIQUAD81 and the results are shown in Table 3. Each titration was treated individually, and the glycine pK_a's, K_w, and E° were kept constant at the values determined previously (see the previous section). Later, E° was refined as it was for the pK_a determinations. In solutions with glycine:nickel ratios of less than 2:1, the formation constant for the NiL_3 species could not be determined but rather was fixed at an average value determined from titrations where the glycine:nickel ratio was greater than 2:1. Figure 6 shows titration curves collected from titrations of solutions of glycine:nickel ratios of 1:1, 2:1, and 4:1 along with the theoretical curves calculated using the formation constants determined by MINIQUAD81.

Table 3.

Formation Constants Determined for Nickel-glycine Complexes

DATE/FILE	L:M Ratio[a]	Sum.Sq.[b]	$\log\beta_{110}$	$\log\beta_{120}$	$\log\beta_{130}$
MAR15001	2:1	1.5	5.61(3)[c]	10.41(4)	----[d]
MAR14001	4:1	5.5	5.57(5)	10.47(5)	13.70(14)
MAR14003	3:1	1.5	5.65(4)	10.56(5)	13.31(27)
MAR11005	1:1	2.6	5.79(2)	10.32(9)	----
APR22002	3:1	0.08	5.61(2)	10.38(2)	13.85(4)
APR22004	2:1	0.8	5.51(6)	10.47(19)	----
APR22006	0.7:1	0.04	5.60(3)	10.41(31)	15.50(72)
AVERAGE[e]	---	----	5.63(8)	10.41(9)	13.6(2)
WT'D. AVG.[f]	---	----	5.61(3)	10.41(4)	13.8(1)
LITERATURE[g]	---	----	5.64(7)	10.39(9)	13.9(2)

[a]Ratio of ligand (L) to metal (M) in solution.
[b]10^6xSum of squares of the residuals, defined Ch.II.
[c]Numbers in brackets refer to the standard deviation in the last digit of the result
[d]Species rejected by MINIQUAD81: low ligand:metal ratio
[e]Simple mean and standard deviation of individual results, not considering standard deviation of each result.
[f]From equations 27 and 28.
[g]Reference 63.

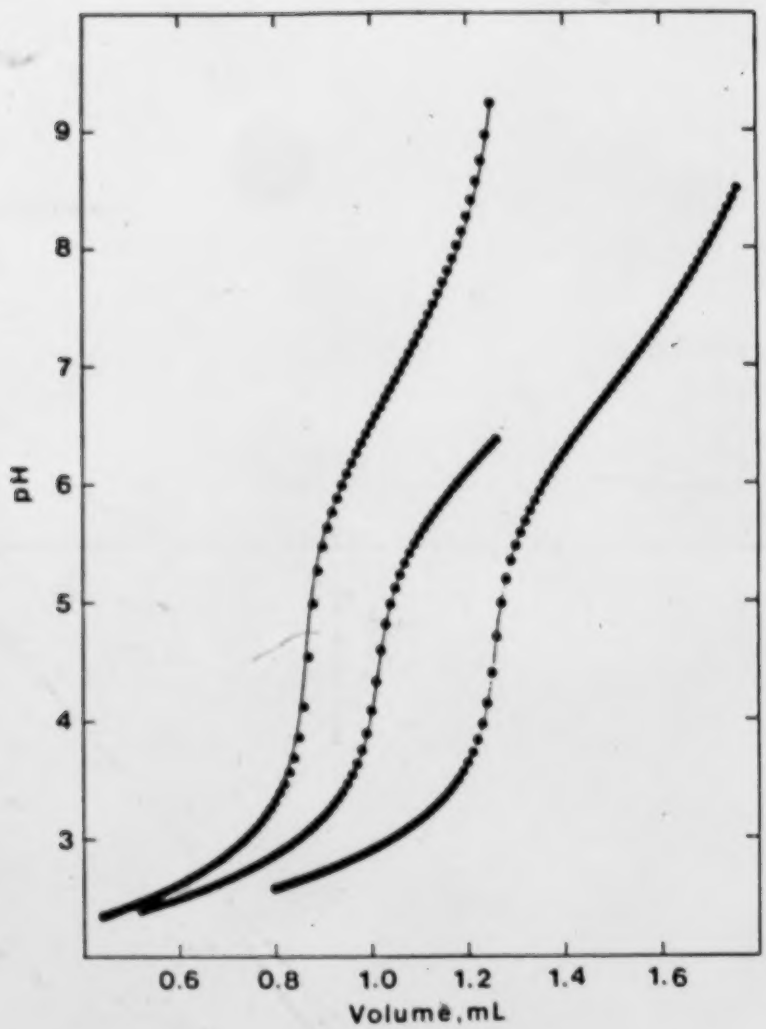


Figure 6. Typical experimental (●) titration curves and corresponding theoretical (—) titration curves for solutions containing glycine and nickel(II) in ratios of (left to right) 1:1, 2:1 and 4:1.

C. Discussion

Titrations are still an important practice in both commercial and research laboratories. Sometimes this is because a direct instrumental method is not available, but in any case, it is generally accepted that better precision is possible with titrations. Unfortunately, manual titrations are time-consuming and the results ultimately depend on the skill of the technician.

With the advent of affordable technology the move to automation has become very attractive. Indeed, in fields involving equilibrium studies, where hundreds of data points might be collected in a single titration, the automation of the titration system has afforded the scientist the ability to collect data which is more free of human error. It has also given him more time for data evaluation and for other duties and experiments.

Most of the many automatic titration systems developed have been of either of two types. First, they were of the dedicated hardware variety, possibly having some software control (73,74). These were the original autotitrators and were generally developed for commercial purposes. They had little or no capability for operator control over the course of the titration, and most of these systems were used solely for end point titrations.

Secondly, there have been the minicomputer- (75-77) and microprocessor-controlled (78-84) titration systems. These

afforded the user much more control over the titration conditions and progress, but their set-up often involved the development of complicated and expensive instrument interfacing. A bonus for these systems was that they could be used for things other than titrations, such as for word processing, graphics, titration simulations, and simple calculations. The extent of the calculations viable on the microprocessor, however, was limited chiefly by the available memory. Indeed, the first microprocessor-controlled autotitrators seemed to be not much more than expensive models of those of the first generation.

Recently, the necessary interfacing and memory expansion technology has become commercially available, making the microprocessor-controlled titration systems more attractive. It is now possible for nearly all the titration calculations, even those previously done only with mainframe computers, to be done with a microprocessor. This saves time and money in that data need not be transferred to another computer before processing, and the access to a large computer and the interfacing for that operation is no longer required.

The titration system described in this thesis was devised to permit accurate determination of formation and ionization constants in various chemical systems, and also to allow the concentrations of solution components to be determined. The computer and its programs provide control over several titration parameters, including the amount and

rate of titrant addition, recognition of hardware error, and reading and recording of the titration data. The Orion 701A pH meter was used because it is equipped with a "data ready" signal which is used to eliminate the collecting of nonsense signals from an unstable meter. The optical isolator aids in eliminating electrical noise. The glass titration cell is temperature controlled for its entire length to counteract the effects of varying temperature along the length of the electrodes. The glass indicating electrode is of the usual type but is of a high resistance glass and has been found to give reliable pH readings in the range of 1 to 14. A double junction S.C.E. reference electrode which contacts the test solution by means of a ground glass junction rather than a porous frit was used to help reduce and make constant the liquid junction potentials. Its outer cell is filled with a solution of the same ionic strength as the test solution to further reduce the liquid junction potential and to reduce the effect of leakage of electrode filling solution into the test solution.

As with all new systems, this one was not without its problems. Firstly, it seems as though very strongly basic (greater than 2 M) titrant will react with the tantalum buret plunger and form a white precipitate on it. No appreciable reaction takes place if the titrant is dilute enough (less than 1 M).

Secondly, it was found that if the burette tip was

moved up or down, the level of titrant at the tip would change. Thus, when the tip was inserted in the solution to be titrated, air could be drawn up into it or titrant solution could be expelled. When standardizing solutions, this was especially important since it introduced error in the volume of titrant delivered from the burette and so changed the concentration value. For a precision of 1 ppt with an equivalence point of 0.8 mL. (usually used), errors of more than 0.8 microliters cannot be tolerated. This problem was solved by keeping the tip at one level and raising and lowering the test solution. However, some data were collected before this was discovered, and it probably contributes to the relatively large deviations seen in the final formation constants and ionization constants.

Another potential problem is the "sucking-back" of titrant through the burette stopcock into the titrant reservoir. It was found that when the titrant reservoir was air-tight, i.e. to exclude carbon dioxide, removal of titrant to fill the buret created a partial vacuum in the titrant reservoir. Even though the buret stopcock was closed after filling the buret, it was not as tight as it should have been and consequently titrant could be "sucked-back" to the reservoir through it. This had the effect of causing badly oscillating slopes (the relative change of pH. or mV. with volume of titrant added as described in Chapter II) near the endpoint of a strong acid or base standardization titration, and was sometimes so bad that an

endpoint had to be picked on the basis of the pH or mV value at which it was expected to occur. This problem was solved by simply opening the titrant reservoir to equalize the pressure before each titration. Once the problem was solved, smooth slope changes were obtained when small volumes of titrant were added.

One of the purposes of doing these experiments was to validate the use of the IBM PC-based automatic titrator for determining formation constants of metal-ligand complexes and ionization constants of ligands. To this aim, it was a success since it is easy to see that the values obtained for the formation constants of the Ni^{2+}-glycine complexes shown in Table 3 fall well within the range reported in the literature (63) (except for that of the 3:1 complex but since there are only four sets of data to estimate this by and a few were not of a high enough metal-ligand ratio to be useful, this difference is not considered significant for the evaluation of the titrator). The results indicate that the automatic titrator provides reliable data for use in formation and ionization constant determinations. It is simple to operate, does not need to be constantly attended, and will give good final results.

It was also found in this study that the Ni(II)-glycine system is a good one to use in evaluating automatic titrators since it is well behaved and well documented.

The experiments presented in this chapter of this

thesis were done to investigate the effectiveness of the autotitration system described in Chapter II, that consisting mainly of commercially available components, for determining equivalence points and equilibrium constants and to prove the nickel(II)-glycine complex to be a good one to use to evaluate other such titration apparati. Having been evaluated by several independent laboratories and found to give consistent formation constants on many systems, it would seem that the nickel(II)-glycine system is a good one for this task.

D. Further Considerations of the Nickel-Glycine System

The autotitrator was evaluated by determining the formation constants of Ni^{2+}-glycine complexes assuming that only the pre-established equilibria (63) are important. In considering the possible chemistry involved, it seemed likely that some previously unreported species might also form as was suggested by Williams and Corrie in the above study.

The Ni^{2+}-glycine titration data collected for the titrator evaluation were rerun with MINIQUAD81 with several possible protonated and hydroxy species included in the model. Species which were repeatedly rejected by MINIQUAD81 or having greatly varying values for formation constants

were assumed to be absent, or at least in very low abundance over the pH range studied (pH 2 to 9.5). Additional complexes not considered in evaluating the titration data to obtain the constants listed in Table 3 but which were accepted by MINIQUAD°1 when the data were rerun were the species Ni^{2+}(glycine)H^{+} and Ni^{2+}(glycine)OH^{-}, designated by the MINIQUAD°1 formation constants β_{111} and β_{11-1}, respectively. Table 4 shows the results when these species were included in the model and the formation constants of all species were re-evaluated.

Again, since the data was not collected with the purpose of determining the best formation constants for the Ni(II)-glycine system, the values above should not be taken as reference values. Further experiments will be done at a later date to obtain these values. They are reported only to indicate the possible presence of species previously considered unimportant or not considered at all by other investigators. The improvement of the fit of the data to the theoretical model shows that these species are probably present and, in my opinion, that the chemical model is more complete when they are included.

Table 4.

Redetermined Values for the

Ni^{2+}-Glycine Formation Constants

Data File	$\log\beta_{110}$	$\log\beta_{120}$	$\log\beta_{130}$	$\log\beta_{11-1}$	$\log\beta_{111}$
MAR15001	5.59(1)[a]	10.21(1)	13.61(4)	-----	10.76(5)
MAR14001	5.598(2)	10.280(3)	13.71(7)	-3.31(3)	-----
MAR14003	5.604(4)	10.295(4)	13.81(1)	-4.05(3)	-----
MAR14004	5.565(4)	10.36(1)	[13.80][b]	-3.663(3)	-----
MAR11005	5.524(4)	10.13(1)	[13.80]	-4.11(4)	-----
APR22002	5.611(7)	10.343(7)	13.76(2)	-3.56(3)	10.62(8)
AVERAGE	5.58(3)	10.26(9)	13.72(7)	-3.7(3)	10.69(7)
WT'D AVG[c]	5.585(6)	10.285(8)	13.79(4)	-3.70(3)	10.72(7)
LITERATURE[d]	5.64(7)	10.39(9)	13.92(2)	-----	-----

[a]Numbers in round brackets refer to standard deviation.

[b]Numbers in square brackets refer to values kept constant during the MINIQUAD81 refinement of the formation constants.

[c]Equations 27 and 28.

[d]Reference 63.

CHAPTER IV

The Formation Constants of Zinc(II) Complexes of Selected Small Peptides

A. Introduction

This chapter presents the results of equilibrium studies done to determine formation constants of zinc(II) complexes of three small, histidine-containing peptide ligands: glycyl-L-histidine, L-alanyl-L-histidine, and glycyl-L-histidyl-L-lysine.

For each ligand, the results of the potentiometric determination of the ligand's acid dissociation constants are first presented. Then, the results of ^1H NMR experiments on solutions containing metal and ligand are described. Following that are the results of the metal-ligand potentiometric experiments and a section comparing the ^1H NMR and potentiometric results. The chapter concludes with a discussion of all the results and their implications.

B. Results

1. Glycyl-L-Histidine Complexes

a. The Acid Dissociation Constants of Glycyl-L-Histidine

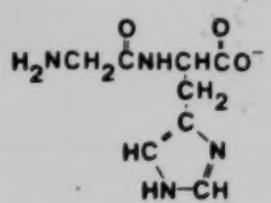
Fully protonated glycyl-L-histidine is a tribasic acid having the following dissociation relationships:

$$AH_3^{2+} \rightleftharpoons AH_2^{+} + H^{+} \qquad (42)$$

$$AH_2^{+} \rightleftharpoons HA + H^{+} \qquad (43)$$

$$HA \rightleftharpoons A^{-} + H^{+} \qquad (44)$$

where A represents the fully deprotonated glyhis molecule whose structural formula is shown below.



The macroscopic proton dissociation constants, K_a's, are defined by the following expressions:

$$K_{a1} = \frac{[H^{+}][AH_2^{+}]}{[AH_3^{2+}]} \qquad (45)$$

$$K_{a2} = \frac{[H^+][AH]}{[AH_2^+]} = \frac{[H^+]^2}{K_{a1}[AH_3^{2+}]} \quad (46)$$

$$K_{a3} = \frac{[H^+][A^-]}{[AH]} = \frac{[H^+]^3}{K_{a1}K_{a2}[AH_3^{2+}]} \quad (47)$$

K_{a1}, K_{a2} and K_{a3} were determined in the same manner as was used for the glycine K_a determinations, that is by MINIQUAD81 processing of potentiometric titration data. Solutions were maintained at a temperature of 25° Celsius throughout each titration. They were of 0.300 molal $(K^+)NO_3^-$ ionic strength and contained one percent D_2O for consistency with the ^1H NMR experiments. The concentration of glycyl-L-histidine was about 0.0187 molal and was standardized as will next be described. Further experimental details are discussed in Chapter II.

The concentration of the glyhis solution was determined by fitting potentiometric titration data with MINIQUAD81 as described in Chapter II. The values used for the constants pK_w and E° were previously determined from KINET treatment of solvent titration data. The E° and pK_w values used in these calculations are shown in Table 5 where the values were weighted as shown by equations 27 and 28. The value used for pK_w was 13.745(5), which was the weighted average of 5 values obtained from the solvent titrations. Since the E° value for the electrode system changed from day to day, possibly depending on the level of filling solution present in the outer compartment of the reference electrode

Table 5.

E° and pK_w Values as Determined by KINET Treatment of Solvent Titration Data[a]

DATE/FILE	E° (mV)	pK_w
JUN09002	406.0(2)	13.728(4)
JUN13004	406.2(3)	13.775(6)
JUN15001	410.5(2)	13.751(4)
JUN15002	410.3(1)	13.745(3)
JUN15003	410.1(2)	13.743(5)
JUN17002	410.82(2)	13.9(7)[b]
JUN20001	410.5(2)	13.6(4)[b]
JUN21001	408.1(2)	13.6(5)[b]
AVERAGE	-------	13.75(1)
WEIGHTED AVERAGE[c]	-------	13.75(1)

[a]Numbers in brackets represent standard deviations in the least significant digits of the preceeding values.
[b]Values not included in calculating the average pK_w since there were too few data points in the high pH region of these titrations to accurately determine this parameter.
[c]Equations 27 and 28.

and the rate at which it could leak out, the value used in the MINIQUAD81 calculations was obtained by interpolation of those for the various solvent titrations.

The fits of the resulting curves to the titration data, as indicated by the sum of squares of the residuals parameter defined in Chapter II, were then monitored as the glyhis concentration was varied. The principle behind this is that a more correct value for the mmoles of ligand should result in a better fit of the titration data to the simulated titration curve. In fact, as the mmoles of ligand is varied, there does occur one value which results in the best fit of the data to the theoretical curve, as shown in Figure 2. When the mmoles of ligand is increased or decreased from this value, the sum of squares of the residuals increases. In this manner, a glyhis concentration of 0.01858 M with an average deviation of 0.00003 M was obtained and used in subsequent calculations.

Once the glyhis concentration was determined, MINIQUAD81 was used to obtain its acid dissociation constants from the titration data. In treating the data, the E° parameter was first made a variable. Since the addition of ligand caused a change in the solvent solution's total ionic strength, even if only slightly, it was expected that the E° value might change from that determined by KINET treatment of solvent titration data. It was found that the final E° obtained differed by little, typically by less than 2 mV, from that previously determined. Then, the optimized

E° value was kept constant and the ligand association constants were rerefined. The results are listed in Table 6. Values found in the literature (15,34) are included for comparison. All sum of squares of the residuals were between 10^{-9} and 10^{-10}, indicating, though somewhat arbitrarily as discussed in Chapter II, a very good fit of the experimental data to the titration curve calculated using the final association constants. Typical titration curves and those calculated from the resulting MINIQUAD81 association constants are shown in Figure 7.

b. The Glycyl-L-Histidine Complexes of Zinc

i. ^1H NMR Experiments

The ^1H NMR potentiometric titration experiments were carried out as described in Chapter II. Ligand solution containing 0.015 M glyhis and metal solution containing 0.0099 M zinc were used to prepare test solutions. The ligand to metal ratios of the test solutions were 2:1 or 1:1, having ligand concentrations of 0.001 to 0.010 molar and metal concentrations of between 0.001 and 0.005 molar were used. Solution ionic strength was maintained at 0.100 molar with $NaNO_3$ and was one percent in D_2O to provide a lock signal for the ^1H NMR spectrometer. The solution pH was adjusted by titration with a concentrated solution of

Table 6.

Glycyl-L-Histidine Proton Dissociation Constants

DATE/FILE	Sum.Sq.[a]	pK_{a1}	pK_{a2}	pK_{a3}
JUN03003	6.18	2.564(8)	6.756(2)	8.137(2)[b]
JUN09003	3.13	2.610(4)	6.770(1)	8.133(1)
JUN10001	5.48	2.586(4)	6.770(2)	8.144(1)
JUN13002	4.76	2.579(5)	6.776(3)	8.141(2)
JUN14003	0.14	2.597(1)	6.769(6)	8.134(3)
JUN17003	0.25	2.602(7)	6.780(4)	8.148(2)
JUN20004	1.56	2.564(5)	6.756(2)	8.127(1)
JUN21003	4.53	2.600(3)	6.792(2)	8.165(1)
WT'D AVERAGES[c]	----	2.61(1)	6.77(1)	8.14(1)
Lit.1[d]	----	2.51	6.77	8.22
Lit.2[e]	----	-----	6.79	8.20

[a] 10^{9} x Sum of squares of the residuals, defined Ch.II.
[b] Values in brackets represent the standard deviation in the last significant digit.
[c] From equations 27 and 28.
[d] Reference 34: 25 °C., 0.2 molar KCl.
[e] Reference 15: 25 °C., 0.16 molar KCl.

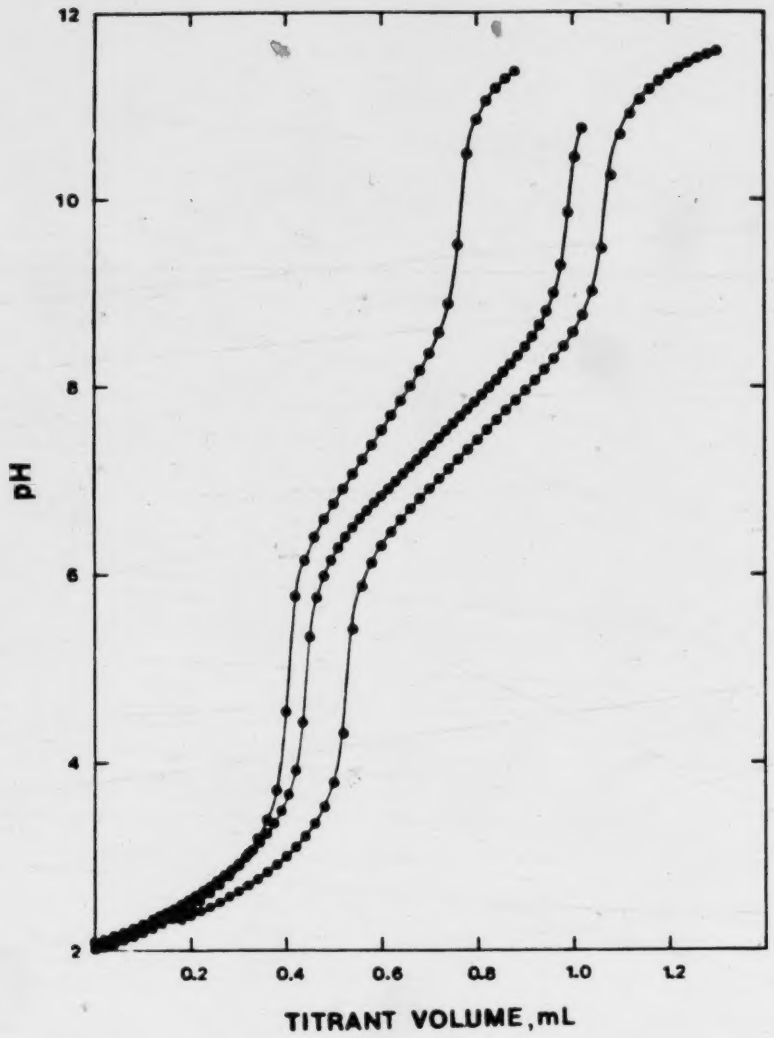


Figure 7. Typical experimental (●) titration curves and corresponding theoretical (—) titration curves calculated using the pK_a's shown in Table 6 for solutions containing 0.010 to 0.018 molal glycyl-L-histidine. Every second data point is plotted.

NaOH or HNO_3.

First, the ligand was titrated alone in solution and the resonances for the C_2H and C_4H protons of the imidazole portion of the molecule were monitored by ^1H NMR. As shown in Figure 8, both resonances are singlets in the 6 - 9 ppm region of the ^1H NMR spectrum. The C_2H proton has a chemical shift of about 8 ppm at low pH where the imidazole group is protonated while the C_4H proton resonance is at about 7 ppm at low pH. As the pH of the solution is raised to within about 1 pH unit of the equivalence point pH for the titration of the acidic proton residing on the imidazole group, the chemical shift of these protons begins to move upfield of their initial positions. This movement accelerates on further pH increases until the pH is past that of the equivalence point for the titration of the proton from the imidazole group. As the pH is increased further, the rate of change in the chemical shifts of these resonances decreases until they become constant at about 7 and 6 ppm respectively. The intensities of the resonances remain fairly constant throughout the titration. The result is a pair of smooth sigmoidal titration curves of chemical shift vs pH, illustrated in Figure 9, which indicates that the glyhis is in fast exchange between its various protonated forms. In following discussions, these resonances will be referred to as free ligand or free imidazole resonances.

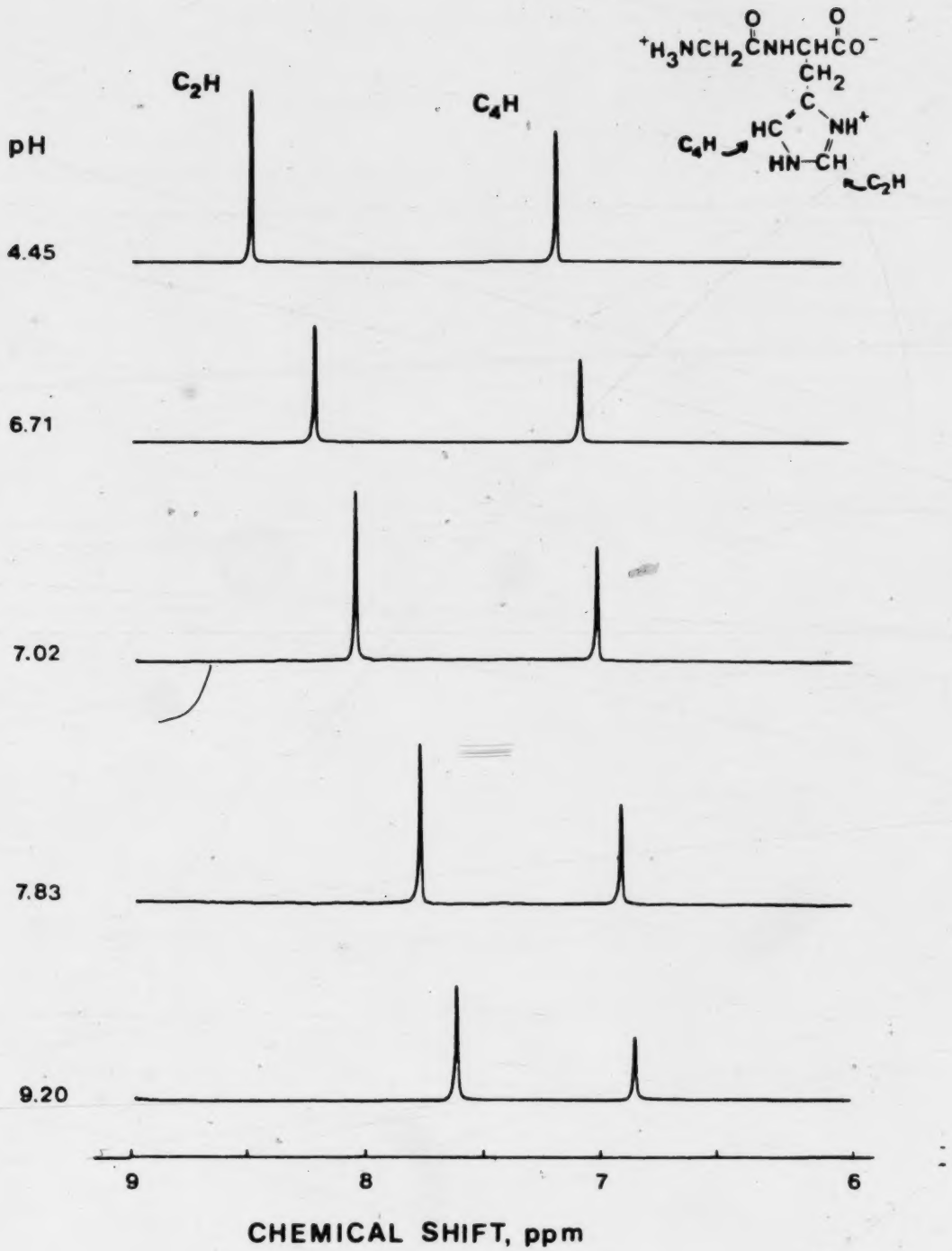


Figure 8. ^{1}H NMR spectra of a solution of 0.005 molal glycyl-L-histidine at several pH values.

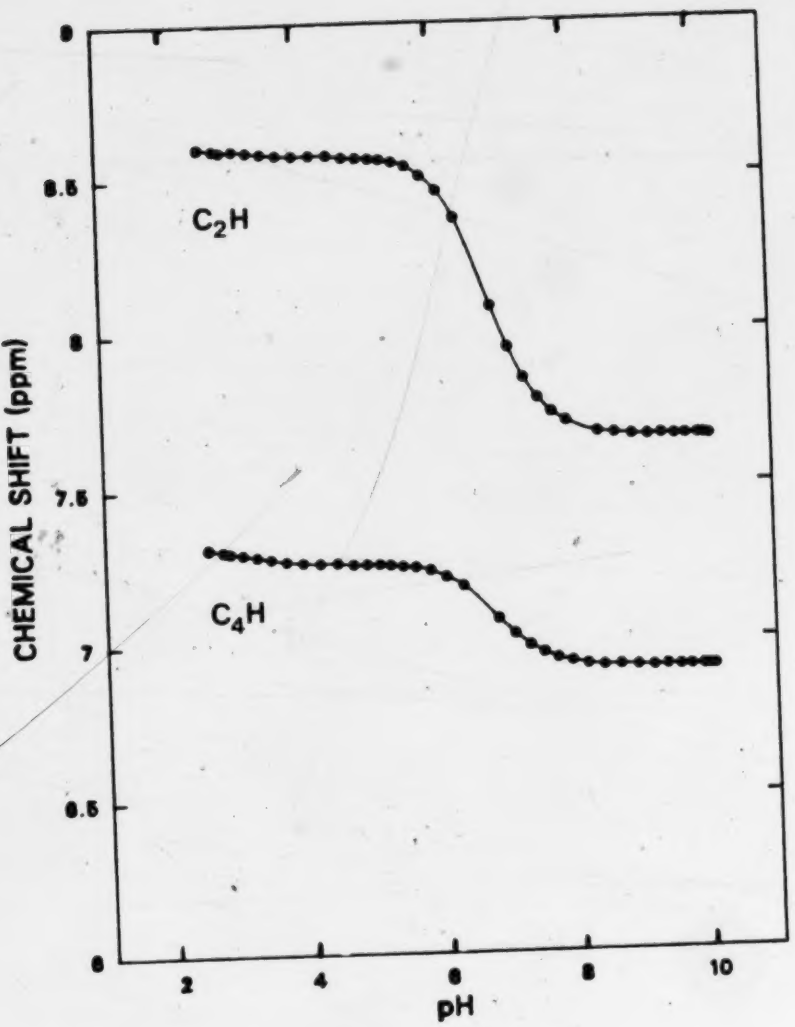


Figure 9. Chemical shift of the imidazole C_2H and C_4H resonances of glycyl-L-histidine as a function of pH for a solution of 0.005 molal glycyl-L-histidine.

Then the ^1H NMR measurements were repeated with ligand in the presence of zinc. In all instances, on reaching a pH of about 7, there appeared two additional singlet resonances in the 6 to 9 ppm region of the spectrum where the free imidazole C_2H and C_4H resonances are found. The two additional resonances were of approximately equal intensity. At pH 4, one was located about 0.4 ppm upfield of the free C_2H resonance while the other was found about 0.4 ppm upfield of the free C_4H resonance. To illustrate, spectra taken at several pH levels are shown in Figure 10.

As the pH was increased, the free imidazole resonances shifted upfield due to the titration of the imidazolium proton, while the chemical shifts of the new resonances remained fairly constant. This constancy of the chemical shift of these resonances indicates that they do not arise from the titration of protons from the ligand since the chemical shifts of the resonances of a molecule in exchange between protonated and deprotonated forms is pH dependent. This behavior is, however, consistent with that in which the ligand is in a strong complex. The separate resonances also indicate slow exchange of the ligand between this complexed form and that in which the ligand is free. A structure has been proposed (32-34) for a Zn(II)-glyhis complex which involves the deprotonation of the amide nitrogen. Such a complex would be expected to exchange very slowly on the NMR time scale. In the interest of clarity, in following discussions, these additional resonances of constant

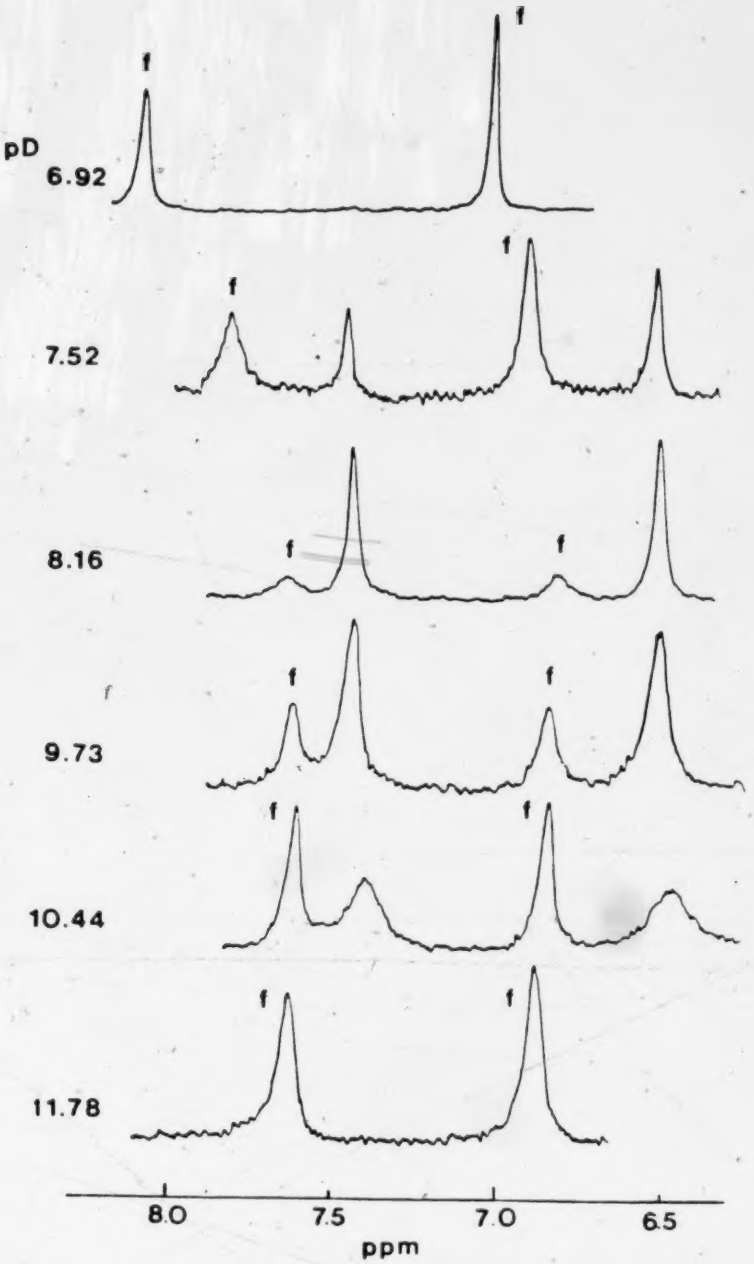


Figure 10. ^{1}H NMR spectra of a solution of 0.005 molal zinc(II) and 0.005 molal glycyl-L-histidine at several pH values. Resonances labelled 'f' are due to 'free' glycyl-L-histidine, defined in the text.

chemical shift will be referred to as being complexed ligand or complexed imidazole resonances.

In addition, the free imidazole resonances of glyhis in solutions containing zinc were found at a chemical shift upfield from the imidazole resonances of glyhis in solutions of glyhis alone, and the difference in chemical shift for the resonances in the two solutions increased as the pH was raised. To illustrate, chemical shift data for the C_2H and C_4H protons of glyhis alone and for a solution of $Zn(NO_3)$- and glyhis are shown as a function of pH in Figure 11.

The intensities of the free and complexed imidazole C_2H and C_4H resonances were measured as described in Chapter II. The intensities of both the complexed imidazole peaks increased at about the same rate as the intensities of the free imidazole peaks decreased. Typical relative intensity behavior of the complexed and free C_2H resonances of the imidazole of glyhis is shown as a function of pH for two different ligand to metal ratios in Figure 12. The C_4H proton resonances exhibited the same behavior. In the case of a solution of a 1:1 ligand to metal ratio, the imidazole resonances of free glyhis became very broad and almost disappeared as the pH was raised to about 9 but then became more intense as the pH was increased further while at the same time the resonances of the complexed glyhis decreased in intensity. In solutions of a 2:1 ligand to metal ratio, the fraction of the complexed imidazole resonance increased

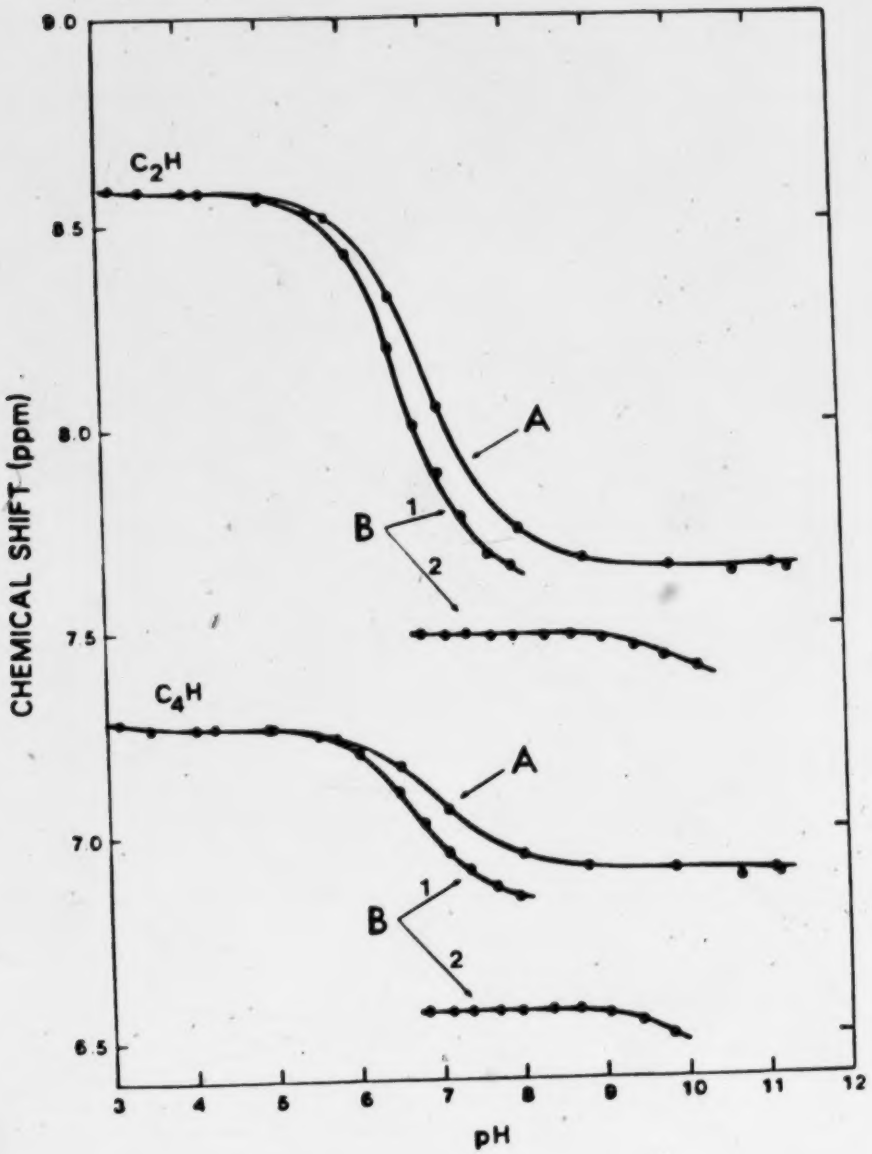


Figure 11. Chemical shift of the imidazole C_2H and C_4H resonances of glycyl-L-histidine (glyhis) as a function of pH in a solution of 0.005 molal glyhis and 0.005 molal zinc(II) compared with those of a solution of glyhis alone; glyhis alone (A), complexed glyhis (B_2) and 'free' glyhis (B_1), defined in the text.

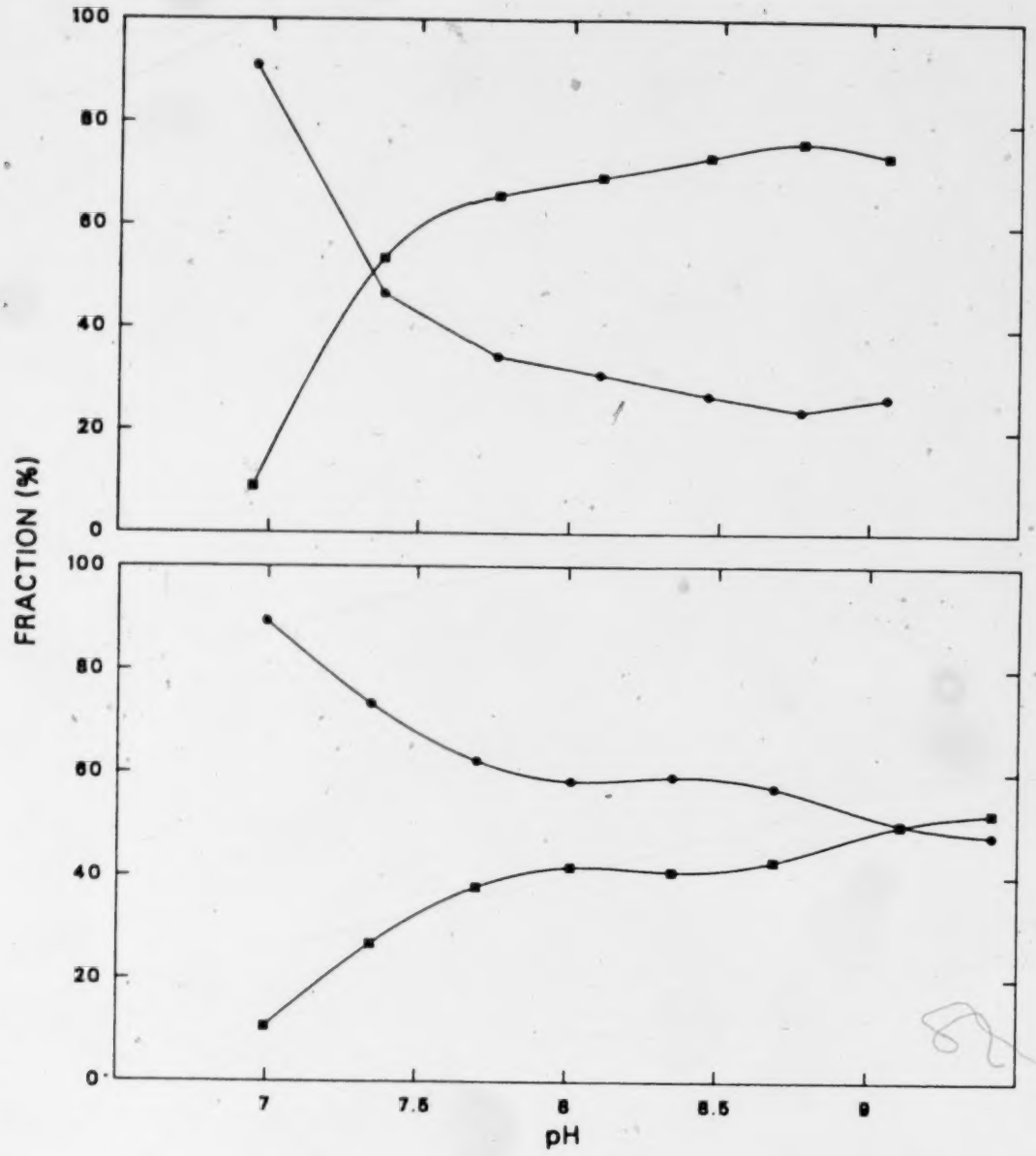


Figure 12. Fraction (%) of the glycyl-L-histidine imidazole C_2H resonances in the 'free' (●) and complexed (■) form as a function of pH for solutions of 0.005 molal glycyl-L-histidine and 0.005 molal zinc(II) (top) and 0.010 molal glycyl-L-histidine and 0.005 molal zinc(II) (bottom).

as the pH was raised but never rose significantly above 50 percent over the pH range studied. As in the 1:1 solutions, the intensity of the complexed resonances decreased above pH 9 but never fully disappeared.

Also, as the pH of solutions of a 1:1 ligand to metal ratio was raised above about 8.5 a white precipitate became visible. This was not seen in solutions containing glyhis and zinc at a ratio of 2:1.

ii. Potentiometric Titration Experiments

The procedure used to collect potentiometric titration data on solutions containing zinc and glyhis involved first titrating a solution containing solvent and glyhis to the second equivalence point of glyhis, and then acidified standard zinc solution was added gravimetrically to the glyhis solution. Solvent was also added, if necessary, to further adjust concentrations and/or to reduce the solution pH. The zinc/glyhis solution was then titrated. The metal ion concentrations ranged from 0.007 to 0.001 molal and the ligand concentrations ranged from 0.013 to 0.002 molal, with final ligand to metal ratios of between 5:1 and 0.7:1. The titrant was 1.0023 M KOH ±0.0002 M average deviation, and contained 1% D_2O and 0.300 molar KNO_3. Data were collected in mV mode to reduce errors introduced by inaccurate pH meter calibration. Only data collected between pH 4 and 9.5

(200 to -200 mV) was used in determining the zinc-glyhis formation constants. That collected above pH 9.5 was subject to error from zinc hydroxide formation and precipitation. This was especially important in solutions of 1:1 ligand to metal ratios or less. The onset of precipitation could be detected prior to being visible by the longer than usual equilibration times and drifting pH meter readings. Further procedural details for these experiments are outlined in Chapter II.

Proton balance calculations, using equations 48 and 49, were done to determine the number of protons titrated from the ligand. As stated in Chapter II, first the solvent solution is titrated, then ligand solution is combined with solvent and the resultant solution is retitrated. Then metal solution is added and the final solution is again retitrated. The protons and base from all these titrations must be counted and equations 48 and 49 are used for this purpose.

$$\text{Theor. } H^+ = Wt_S C_{H+,S} + Wt_L (xC_{L,L} + C_{H+,L}) + Wt_M C_{H+,M} \qquad (48)$$

$$\text{Titrated } H^+ = C_{OH-,T}(V_S + V_{LS} + V_{MLS}) \qquad (49)$$

The symbols V_S, V_{LS} and V_{MLS} represent the volume of titrant (T) used in titrating the solvent, ligand-plus- solvent, and metal-plus-ligand-plus-solvent solutions, respectively. The term Wt indicates the weight of the the individual solutions

involved in the titrations stated above such that the subscripts S, L, and M designate parameters for the solvent, ligand, and metal solutions, respectively. The symbol $C_{a,b}$ refers to the concentration of component a in solution b, using the same subscripts as for the Wt parameter. The concentration of titrant solution is expressed in molar units while that of all other solutions is in molal units, the reason for which is discussed in an earlier section. The symbol x refers to the number of titratable protons on the ligand and, as shown in the previous section of this chapter, there are normally three titratable protons on the fully protonated glyhis molecule, all of which should be completely titrated before pH 9.

The results in Table 7 indicate that an additional proton is titrated from the ligand molecule when zinc ion is present in solution. Even in solutions containing less glyhis than metal, more protons were titrated than were expected, and the amount corresponds to approximately one extra proton per glyhis molecule.

The data was then processed with the program MINIQUAD81 as described in Chapter II using values for K_w, E°, and the concentrations of glyhis and zinc determined from previous experiments. Those solutions having less than a 1:1 ligand to metal ratio were not considered in these calculations. Initially, E° was fixed at a value determined from solvent titrations, then was refined to account for a change in E° due to the combination of different solutions (even though

Table 7.

Proton Balance Calculations

from Zn(II)-Glycyl-L-Histidine Titrations

DATE/FILE	[L]:[M] (RATIO)	TITRATABLE[a] MMOLES H^+	MMOLES H^{+b} TITRATED	DIFFERENCE	MMOLES Zn^{2+}	DIFFERENCE/ MMOLES Zn^{2+}
JUN03004	2:3	0.855	1.266	0.411	0.424[c]	0.970[d]
JUN09004	1:1	0.962	1.614	0.652	0.569	1.145
JUN10002	2:3	1.348	1.744	0.396	0.499[c]	0.793[d]
JUN13005	5:1	2.424	2.595	0.171	0.145	1.181
JUN14004	0.7:1	0.529	0.687	0.157	0.193[c]	0.813[d]
JUN17004	3:1	0.764	0.847	0.083	0.080	1.035
JUN20003	2:1	0.739	0.837	0.098	0.108	0.909
JUN20005	1:1	0.989	1.273	0.284	0.284	1.000
JUN21003	2.5:1	0.752	0.872	0.120	0.123	0.978

[a]Equation 48.

[b]Equation 49.

[c,d]Values represent mmoles glyhis and difference/mmoles glyhis respectively since [glyhis]<[Zn^{2+}]

the solutions were all made to be of as similar an ionic composition as possible), and then was kept constant at this new optimized value.

The potentiometric titration data were then analyzed with MINIQUAD81 to obtain formation constants. The procedure involved determining the best model for the complexation equilibria by running the program in model select mode. The best model was chosen on the basis of the fit of the experimental data to the model as measured by the sum of squares of the residuals parameter. Only species that made sense chemically were included in this process.

The species distribution and titration curve simulation program COMIX was then run with the constants obtained from the above analysis of the data to determine the relative concentrations of the various species included in the model. The formation constants were then further refined with MINIQUAD81 by including only those species found to be at least 5 percent of the total species distribution at some pH between pH 4 and 9.5. The final formation constants obtained are shown in Table 8. As stated in the previous chapter, the nature of the weighting equations explains the relatively small standard deviations seen in the final results compared with those which would be expected from simple, non-weighted calculations. Figure 13 shows typical titration curves along with corresponding curves calculated from the constants in Table 8. A typical species distribution plot for a solution containing zinc and glyhis

Table 8.

Formation Constants of

Zn(II)-Glycyl-L-Histidine Complexes

DATE/FILE	M:L conc.ratio[a]	Sum.Sq.[b]	$\log\beta_{11-1}$[c]	$\log\beta_{110}$	$\log\beta_{111}$	$\log\beta_{12-1}$	$\log\beta_{120}$
JUN13005	2.6:13.0	3.68	-2.32(5)[d]	5.15(1)[e]	10.22(3)	0.90(9)	8.83(8)
JUN17004	0.85:2.34	0.22	-2.14(4)	3.79(2)	11.77(3)	1.28(1)	8.33(3)
JUN20003	1.13:2.52	0.16	-2.75(9)	3.87(2)	11.61(3)	1.671(8)[e]	8.971(7)
JUN20005	3.05:3.33	29.8	-2.04(3)	-------[h]	11.27(6)	1.22(9)	7.5(2)
JUN21003	1.50:3.78	0.23	-3.3(4)	4.04(6)	11.59(2)	1.317(5)	8.777(5)
WT'D AVERAGES[f]	---------	----	-2.14(4)	3.85(2)	11.54(2)	1.31(1)	8.85(1)
LITERATURE[g]	---------	----	-2.75	3.98	10.87	0.37	8.03

[a]Concentrations of zinc (M) and glyhis (L) are in units of mmoles per gram solution.

[b]10^8xSum of squares of the residuals, defined Ch.II.

[c]Symbols defined in section IV B 1 b i of text.

[d]Bracketted numbers are std. dev. of the least sig. figs.

[e]Value rejected by the Q test (68).

[f]Equations 27 and 28.

[g]Reference 34: includes species Zn(glyhisH$_{-2}$), $\log\beta_{11-2}$=-12.66.

[h]Species rejected by MINIQUAD81.

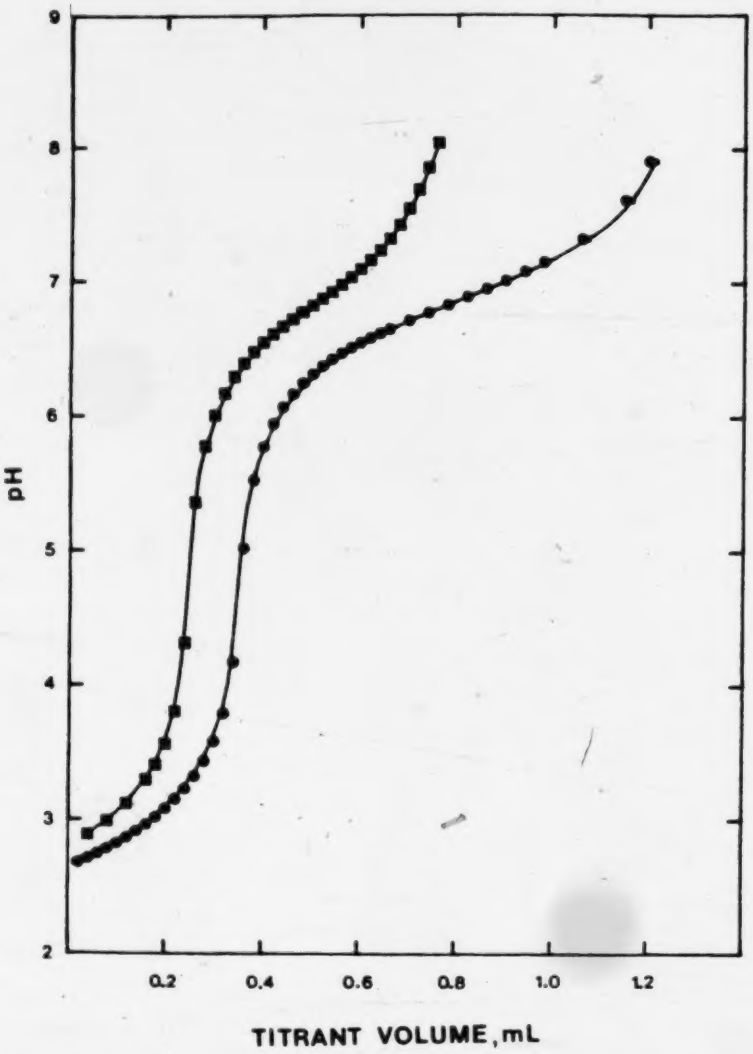


Figure 13. Typical experimental (●■) titration curves and corresponding theoretical (—) titration curves calculated from the formation constants shown in Table 8 for solutions containing glycyl-L-histidine and zinc(II) in ratios of (left to right) 2:1 and 1:1. Every second data point is plotted.

in a 1:1 ratio, calculated from the final formation constants determined for the system, is shown in Figure 14.

Those species found to be significant were Zn(glyhis), Zn(glyhisH), $Zn(glyhis)_2$, Zn(glyhis)OH and $Zn(glyhis)_2OH$. Charges have been omitted for simplicity. In the following sections, they are sometimes referred to by their formation constant designations, i.e. β_{110}, β_{111}, β_{120}, β_{11-1}, and β_{12-1} respectively, where the subscripts represent the stoichiometric coefficients of the components metal, ligand and protons of the complex. A negative value for the protons implies either the presence of OH^- on the molecule or the titration of more protons from the molecule than expected, i.e. protons which would not normally be titrated from the free ligand itself. For example, the species Zn(glyhis)OH could actually be of the form $Zn(glyhisH_{-1})$, where an additional proton has been titrated from the glyhis molecule. Similarily, the species $Zn(glyhis)_2OH$ might also be of the form $Zn(glyhisH_{-1})(glyhis)$. This is discussed further in the discussion section. There is no way to distinguish between the existence of either type of species using MINIQUAD81 alone.

Binuclear species of the type $Zn_2(glyhis)_x$ were repeatedly rejected by MINIQUAD81 and were eliminated from the model.

It was found that the inclusion of $Zn(OH)_x$ species, using formation constants available in the literature (64), improved the fit of the data to the theoretical curve.

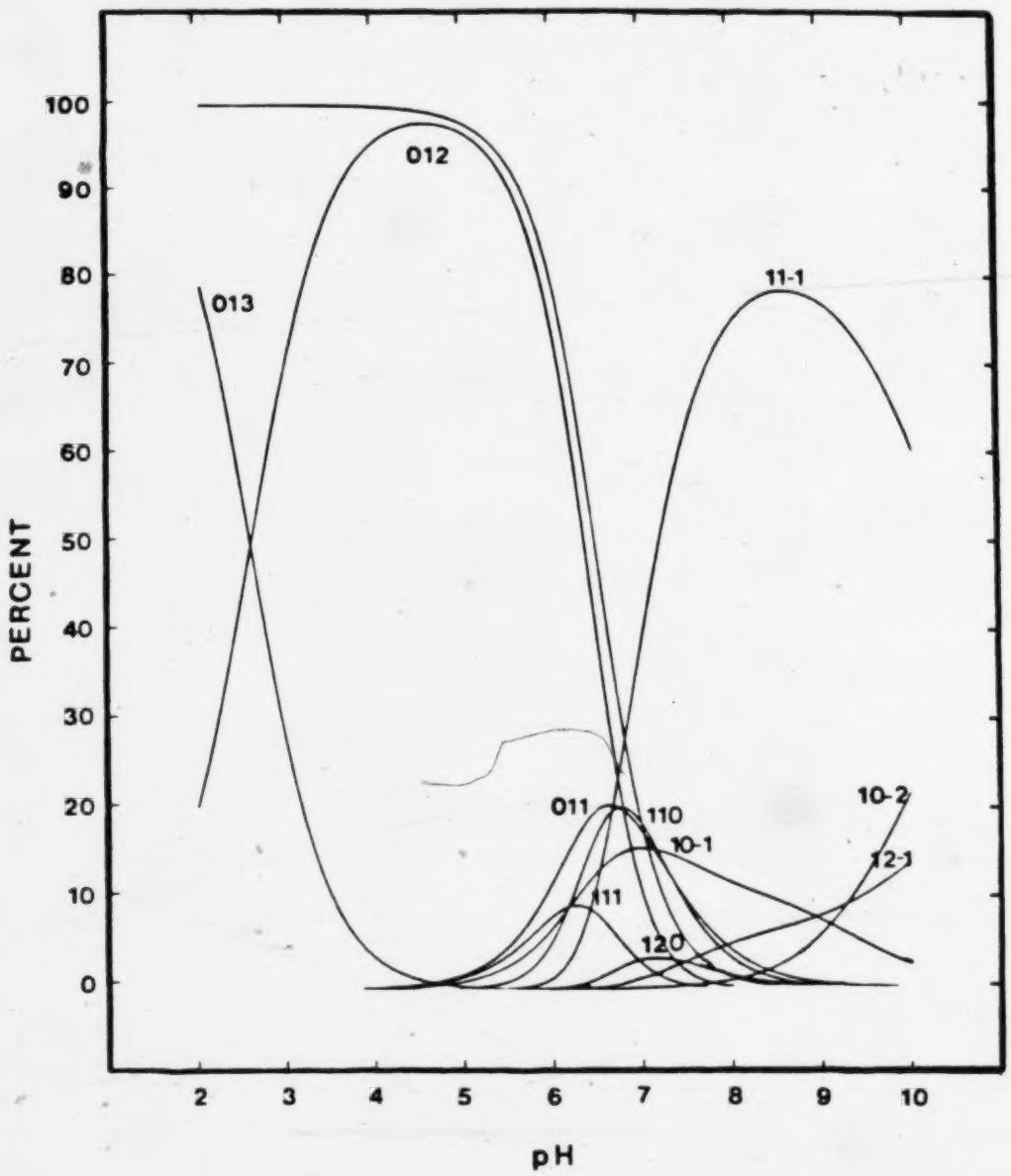


Figure 14. Theoretical Zn(II)-glycyl-L-histidine species distributions calculated using formation constants shown in Table 8 for a solution containing 0.005 molal each of glycyl-L-histidine and zinc(II), reported as percent total glycyl-L-histidine. The curve labels identify species by the subscripts of their MINIQUAD81 formation constants, defined in the text.

COMIX species distribution calculations using formation constants found in the literature revealed that only the species $Zn(OH)^+$ and $Zn(OH)_2$ were in significant abundance (greater than 5 percent of the total zinc concentration) over the pH range 4 to 9.5. To obtain formation constants for these complexes in solutions of ionic strength comparable to that used in this study, aliquots of stock zinc solution were titrated potentiometrically and the data was processed with MINIQUAD81. Values for the overall formation constants for the species $Zn(OH)^+$ and $Zn(OH)_2$ were determined to be 6.62(1) and 11.44(1) respectively and were used in subsequent zinc(II)-ligand formation constant calculations.

c. Comparison of ^1H NMR and Potentiometric Results

The fractions of the total glyhis present in the complex which gives the additional set of slow exchange imidazole resonances (Figure 10) were calculated from the relative resonance intensities by the cut and weigh method from the total weights of the C_2H and C_4H 'free' and C_2H and C_4H complexed peaks respectively. The program COMIX was then used with the formation constants determined for the Zn(II)-glyhis complexes in the previous section to simulate species distributions for the concentrations used in the 1H

NMR titrations. The predicted concentrations were compared with those obtained from the ^1H NMR experiments. Those species formulated as Zn(glyhis)OH or Zn(glyhisH$_{-1}$) and Zn(glyhis)$_2$OH or Zn(glyhis)(glyhisH$_{-1}$) in the evaluation of the potentiometric titration data were assumed to be the complexes giving the slow exchange resonances. All other species involving glyhis, free and complexed to zinc but not the species Zn(glyhisH$_{-1}$) and Zn(glyhis)(glyhisH$_{-1}$), were considered to contribute to the 'free' ligand resonances in the ^1H NMR spectra.

Tables 9 and 10 show the results of these calculations. While the two species distributions did not exactly match, the trend of 'free' ligand abundance over the pH range studied was the same and considering the estimated errors of ±10% in determining the fractional concentrations fromothe areas of these resonances, the agreement is quite good. In all cases, it was seen that the fraction of the ligand in the 'free' form decreased as the pH was increased, reaching a minimum value at about pH 8.5 to 8.8 and then increasing as the pH was raised further. For 2:1 data sets (Table 10), the fraction of the ligand in the free form was at a minimum at pH 9 to 9.4 and then increased on further base addition.

The agreement between the potentiometric titration and ^1H NMR results lends support for the inclusion of all species in the proposed Zn(II)-glyhis model. The nature of the complexes, particularily the Zn(glyhisH$_{-1}$) complex, is considered in detail in the discussion section.

Table 9.

Comparison of Observed[a] and Predicted[b] Percentages of Glycyl-L-Histidyl Ligand in the Free[c] Form as a Function of pH[d]

	CASE 1		CASE 2		CASE 3	
pH	Observed	Predicted	Observed	Predicted	Observed	Predicted
6.9	----------		91		----------	
	----------		87	78	----------	
7.0	----------		----------		72	
	----------		----------		84	71
7.1	74	68	----------		----------	
7.3	----------		----------		53	
	----------		----------		69	55
7.4	55		47		----------	
	65	51	51	50	----------	
7.6	----------		----------		42	
	----------		----------		39	40
7.8	35		34		----------	
	31	34	33	33	----------	
7.9	----------		----------		35	
	----------		----------		28	29
8.1	----------		31		----------	
	----------		45	25	----------	
8.3	----------		----------		24	
	----------		----------		18	22
8.5	----------		27	21	----------	
8.8	----------		24	20	----------	
9.1	----------		26	22	----------	

[a] From ^{1}H NMR experiments.
[b] From COMIX calculations using the formation constants shown in Table 8.
[c] Defined in text.
[d] Equimolal metal and ligand; CASE 1, 0.002 molal; CASE 2, 0.003 molal each; CASE 3, 0.005 molal each.

Table 10.

Comparison of Observed and Predicted Percentages of Glycyl-L-Histidyl Ligand in the Free Form as a Function of pH[a]

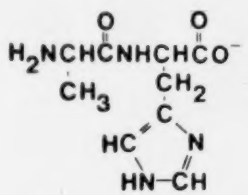
pH	CASE 1 Observed	CASE 1 Predicted	CASE 2 Observed	CASE 2 Predicted	CASE 3 Observed	CASE 3 Predicted	CASE 4 Observed	CASE 4 Predicted
7.0			89	84			89	
7.3			73	75			88	84
7.4							73	
7.7	71		66		74		75	75
7.8	59	64	70	65	75	72	62	
8.0					66		67	65
8.1	63	57	60		66	63	58	
8.3			69	57	59		65	59
8.4	63				59	57	59	
8.5	61	54	68		56		66	55
8.7			68	54	59	54		
8.8	67						57	
8.9	50	53	65		58		60	52
9.0			59	52	60	51		
9.1							50	
9.2	70	52					56	51
9.3			66		60		48	
9.4			67	51	63	51	53	51
9.5	71	52	65		61			
9.6			65	52	63	51		

[a]Definitions same as for Table 9. Molal L:Zn ratios for CASE 1-4: 0.002:0.001; 0.004:0.002; 0.006:0.003; 0.010:0.005.

2. L-Alanyl-L-Histidine Complexes

a. The Acid Dissociation Constants of L-Alanyl-L-Histidine

Fully protonated L-alanyl-L-histidine is a tribasic acid, chemically similar to glycyl-L-histidine. Its acid-base chemistry is described by equations 42 to 44 found in the previous section of this chapter, where A represents the fully deprotonated alahis molecule.



Similarily, the macroscopic acid dissociation constants of alahis are defined by equations 45 to 47.

The experimental conditions for determining these constants were exactly the same as those used in determining the constants of glycyl-L-histidine. Table 11 shows the results of the determination of the concentration of L-alanyl-L-histidine by potentiometric titration and data processing with MINIQUAD81. The average concentration from nine determinations was 0.0244 molal with an average deviation of 0.0002.

While processing the data, the program ACBA became available. As described in Chapter II, ACBA has the capability of refining several titration parameters

Table 11.

MINIQUAD81 L-Alanyl-L-Histidine Proton Dissociation Constants and Concentration Determinations

DATE/FILE	Conc.[a]	sum.sq.[b]	pK_{a1}	pK_{a2}	pK_{a3}
AUG10001	0.02495	93.0	2.54(3)[c,d]	6.69(2)	8.09(1)
AUG10004	0.02476	2.7	2.78(3)	6.77(1)	8.03(1)[c]
AUG23003	0.02456	3.6	2.67(3)	6.71(2)	8.08(1)
AUG23005	0.02472	6.0	2.68(3)	6.71(2)	8.07(1)
AUG24002	0.02421	1.8	2.734(5)	6.768(3)	8.085(2)
AUG24004	0.02428	2.7	2.739(7)	6.764(4)	8.087(3)
AUG25002	0.02431	2.5	2.69(1)	6.743(6)	8.068(4)
SEP15001	0.02404	7.1	2.743(1)	6.768(7)	8.084(4)
WT'D AVERAGES[e]	0.0244(2)	----	2.73(1)	6.76(1)	8.08(1)

[a]Units of concentration are mmoles per gram solution.

[b]10^8 x sum of squares of the residuals, defined in text.

[c]Value rejected by the Q test (68).

[d]Values in brackets represent standard deviations in the least significant digits of the results.

[e]From equations 27 and 28; equal weighting reduces concentration calculations to simple mean and std. dev.

simultaneously with a fairly high degree of reliablilty. The data collected from the alahis titration experiments was reprocessed with ACBA to obtain the alahis pK_a's and concentration simultaneously. The results, shown in Table 12, compare favorably with those obtained with MINIQUAD81. A concentration of 0.0244(2) molal alahis was obtained with MINIQUAD81 while 0.0244(1) molal was obtained with ACBA, the numbers in brackets being the average deviation of the least significant digits of the result. Since ACBA is a much easier program to use than MINIQUAD81 for this purpose, it was used in further applications of this kind, and 0.0244 molal was the value used for the ligand concentration in the Zn(II)-alahis formation constant determinations. Typical titration curves, both experimental and those calculated from the final proton dissociation constants, are displayed in Figure 15.

b. The Formation Constants of L-Alanyl-L-Histidine Complexes of Zinc

i. ^1H NMR Experiments

The experiments were carried out in the same manner as described earlier for the zinc(II)-glyhis system. Solutions with ligand to metal ratios of 2:1 and 1:1 and having ligand concentrations of between 0.005 molar and 0.010 molar

Table 12.

Determination of L-Alanyl-L-Histidine Proton Dissociation Constants and Concentration with ACBA

DATE/FILE	Conc.[a]	Std.Dev.[b]	pK_{a1}	pK_{a2}	pK_{a3}
AUG10001	0.02457	1.8	2.655(3)[c]	6.724(4)[d]	8.077(5)
AUG10004	0.02423	1.1	2.706(4)	6.737(7)	8.049(7)
AUG23003	0.02437	1.5	2.656(6)	6.72(1)	8.07(1)
AUG23005	0.02448	1.8	2.654(6)	6.72(1)	8.06(2)
AUG24002	0.02430	1.0	2.704(2)	6.755(2)	8.081(3)
AUG24004	0.02434	1.1	2.699(2)	6.748(3)	8.075(4)
AUG25002	0.02454	1.6	2.665(4)	6.731(7)	8.076(8)
SEP15001	0.02417	1.1	2.708(2)	6.757(3)	8.085(4)
WT'D AVERAGES[e]	0.0244(1)	----	2.667(3)	6.75(1)	8.08(1)

[a]Concentration in units of mmoles per gram solution.

[b]Standard deviation in result calculated by ACBA.

[c]Omitted from average - not enough data at low pH.

[d]Values in brackets are standard deviations in the least significant digits of the individual results.

[e]From equations 27 and 28; equal weighting reduces concentration calculations to simple mean and std. dev.

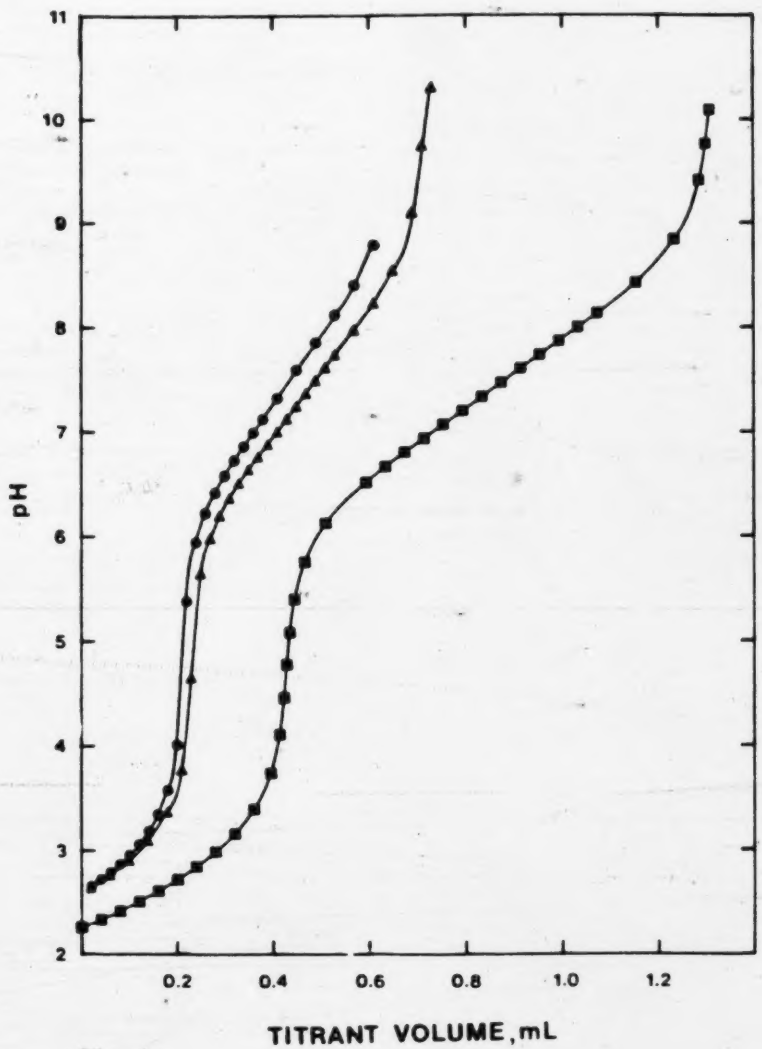


Figure 15. Typical experimental (▲●■) titration curves and corresponding theoretical (—) titration curves calculated using the pK_a's shown in Table 12 for solutions containing 0.010 to 0.024 molal L-alanyl-L-histidine. Every second data point is plotted.

and metal ion concentrations of 0.005 to 0.010 molar were used. The temperature of the probe was 25 (±0.1) °C, and the pH of the test solutions was changed with concentrated NaOH.

Qualitatively, the same observations were made in this chemical system as were made in the zinc(II)-glyhis system. Two additional singlet resonances were seen in the imidazole region of the spectrum after a pH of about 7 was reached. One was located upfield of the free C_2H imidazole resonance, defined in Chapter II, and the other upfield of the free C_4H resonance. Again, the chemical shifts of these new peaks remained fairly constant over the pH range studied while those of the normal imidazole peaks shifted with pH as was observed in the Zn(II)-glyhis experiment. Typical ^1H NMR spectra at several pH values are shown in Figure 16.

Intensity measurements were not made on this system but the qualititive trend of intensity with pH was very similar to that seem with the Zn(II)-glyhis solutions. The intensity of the complexed ligand resonances increased with pH while that of the 'free' ligand resonances decreased until about pH 9 where all resonances became quite broad, then the complex ligand resonances began to disappear and the intensity of the 'free' ligand resonances began to be restored. As with the zinc(II)-glyhis complexes, the intensity of the 'free' ligand resonances nearly disappeared on reaching pH 7 for 1:1 ligand to metal solutions.

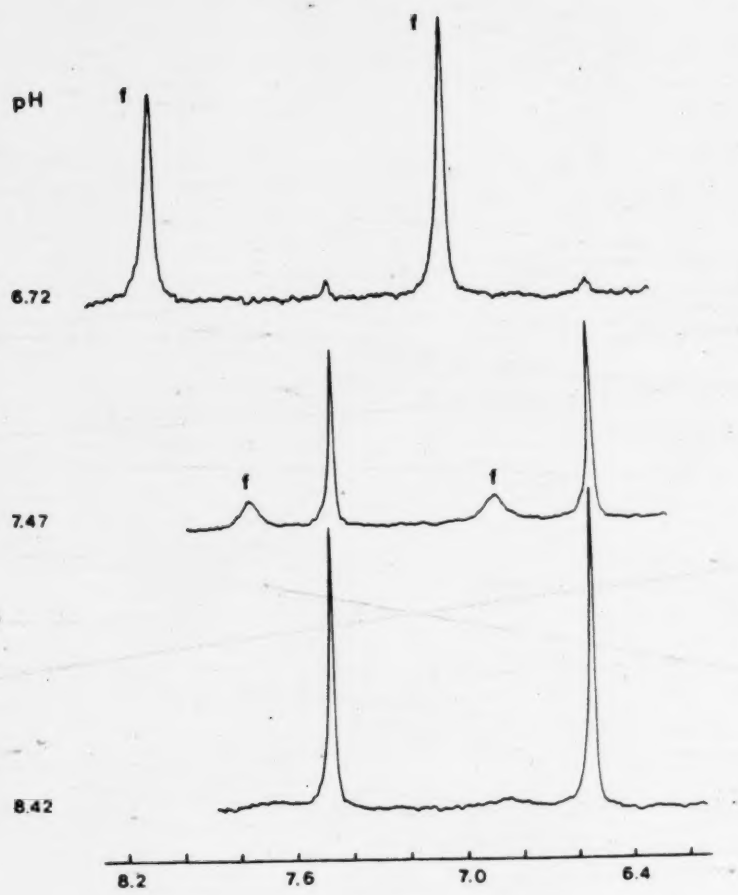


Figure 16. ^{1}H NMR spectra of a solution of 0.005 molal zinc(II) and 0.005 molal L-alanyl-L-histidine at several pH values. Resonances labelled 'f' are due to 'free' L-alanyl-L-histidine, defined in the text.

ii. Potentiometric Titration Experiments

These experiments were performed as outlined in Chapter II of this thesis. All solutions contained 0.300 molal $[K^+]NO_3^-$ and 1% D_2O as in the the Zn(II)-glyhis experiments. The ligand to metal ratios in solution were between 1:1 and 3:1. The concentration of zinc in solution was between 0.00089 and 0.00258 molal while the alahis concentration ranged from 0.00184 to 0.00547 molal. The titrant was 1.0105 ±0.0005 molar KOH. The data were collected in mV mode and only that between pH 4 and 9.5 (200 to -200 mV) was used in determining the zinc(II)-alahis formation constants. The onset of zinc hydroxide precipitation was closely monitored, the titration being terminated if there were long equilibration times or drifting pH meter readings, which indicated such precipitation.

Proton balance calculations were done to see if the number of protons being titrated from the ligand was the same as that expected and the results are presented in Table 13. As observed in the Zn(II)-glyhis system, an average of approximately one additional proton was titrated from the ligand per zinc(II) ion in solution.

The data was then processed to determine the Zn(II)-alahis formation constants. This was done in the same manner as for the data in the zinc(II)-glyhis system, using MINIQUAD81 first in model select mode, then using COMIX to determine the relevant chemical species.

Table 13.

Proton Balance Calculations for the

Zn(II)-L-Alanyl-L-Histidine Chemical System

DATE/FILE	L:M[a] RATIO	TITRATABLE[b] MMOLES H^+	MMOLES H^+[c] TITRATED	DIFFERENCE	MMOLES Zn^{2+}	DIFFERENCE/ MMOLES Zn^{2+}
AUG10002	5.5:2.6	2.637	2.756	0.119	0.1323	0.899
AUG10005	2.1:2.3	1.826	1.985	0.159	0.1562	1.017
AUG23004	2.0:1.8	1.286	1.384	0.098	0.1158	0.846
AUG23006	1.8:0.9	1.416	1.492	0.076	0.0675	1.126
AUG24003	3.3:1.1	1.882	1.986	0.104	0.0947	1.098
AUG25003	2.2:1.2	1.569	1.695	0.126	0.0992	1.270

[a]Ratios of mmolal concentrations of ligand (L) and metal (M).

[b]Equation 48.

[c]Equation 49.

Again, the data was then rerun using MINIQUAD81 with only the species obtained from the above analysis to determine the best constants for the system. Table 14 shows the final formation constants obtained from the calculations and Figure 17 shows typical experimental titration curves and the corresponding theoretical titration curves predicted using the final MINIQUAD81 constants. A typical species distribution plot calculated from the final formation constants for a solution of alahis and zinc in a 1:1 ratio is shown in Figure 18.

Table 14.

Determination of the Formation Constants of

Zn(II)-L-Alanyl-L-Histidine Complexes

DATE/FILE	L:M Ratio[a]	Sum.Sq.[b]	$\log\beta_{11-1}$[c]	$\log\beta_{110}$	$\log\beta_{111}$	$\log\beta_{12-1}$	$\log\beta_{120}$
AUG10002	.005:.0025	42.64	-3.4(2)[d]	3.5(5)	10.53(5)	-0.33(7)	7.79(5)
AUG10005	.002:.002	28.73	-3.37(7)	----------	9.9(5)	0.48(3)	-------
AUG23004	.002:.002	18.75	-3.35(3)	----------	10.3(3)	0.6(1)	-------
AUG23006	.002:.001	2.829	-3.7(3)	----------	10.5(1)	0.2(1)	-------
AUG24003	.003:.001	4.096	-3.17(9)	----------	10.54(6)	-0.20(9)	7.81(4)
AUG25003	.002:.001	5.428	-3.69(1)	----------	10.07(3)	0.51(5)	7.43(8)
WT'D. AVGS.[e]	-------	-----	-3.56(3)	3.5(5)	10.28(5)	0.4(1)	7.77(4)

[a]Molal ratio of ligand (L) to metal (M).

[b]10^{8}xSum of squares of the residuals, defined Chapter II.

[c]Symbols defined in section IV B 1 b i.

[d]Values in brackets are the std. dev. of the least significant digits.

[e]From equations 27 and 28.

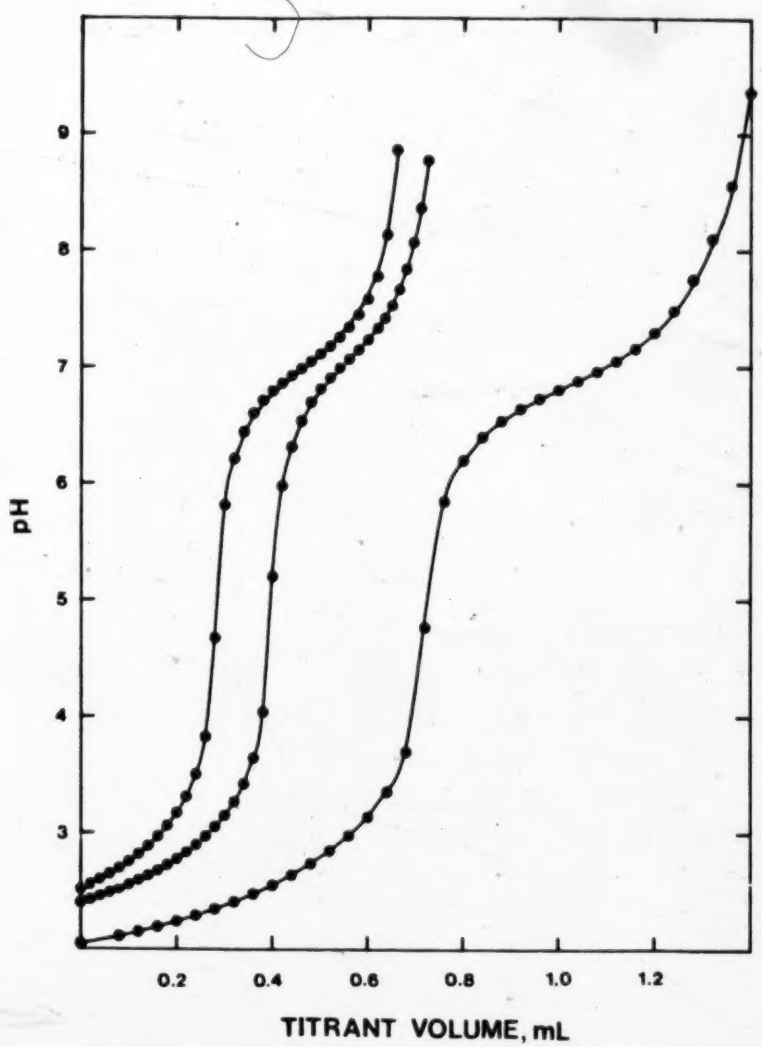


Figure 17. Typical experimental (●) titration curves and corresponding theoretical (—) titration curves calculated from the formation constants shown in Table 14 for solutions containing L-alanyl-L-histidine and zinc(II) in ratios of (left to right) 2:1, 2:1 and 1:1. Each solution contains a different amount of free acid and every second data point is plotted.

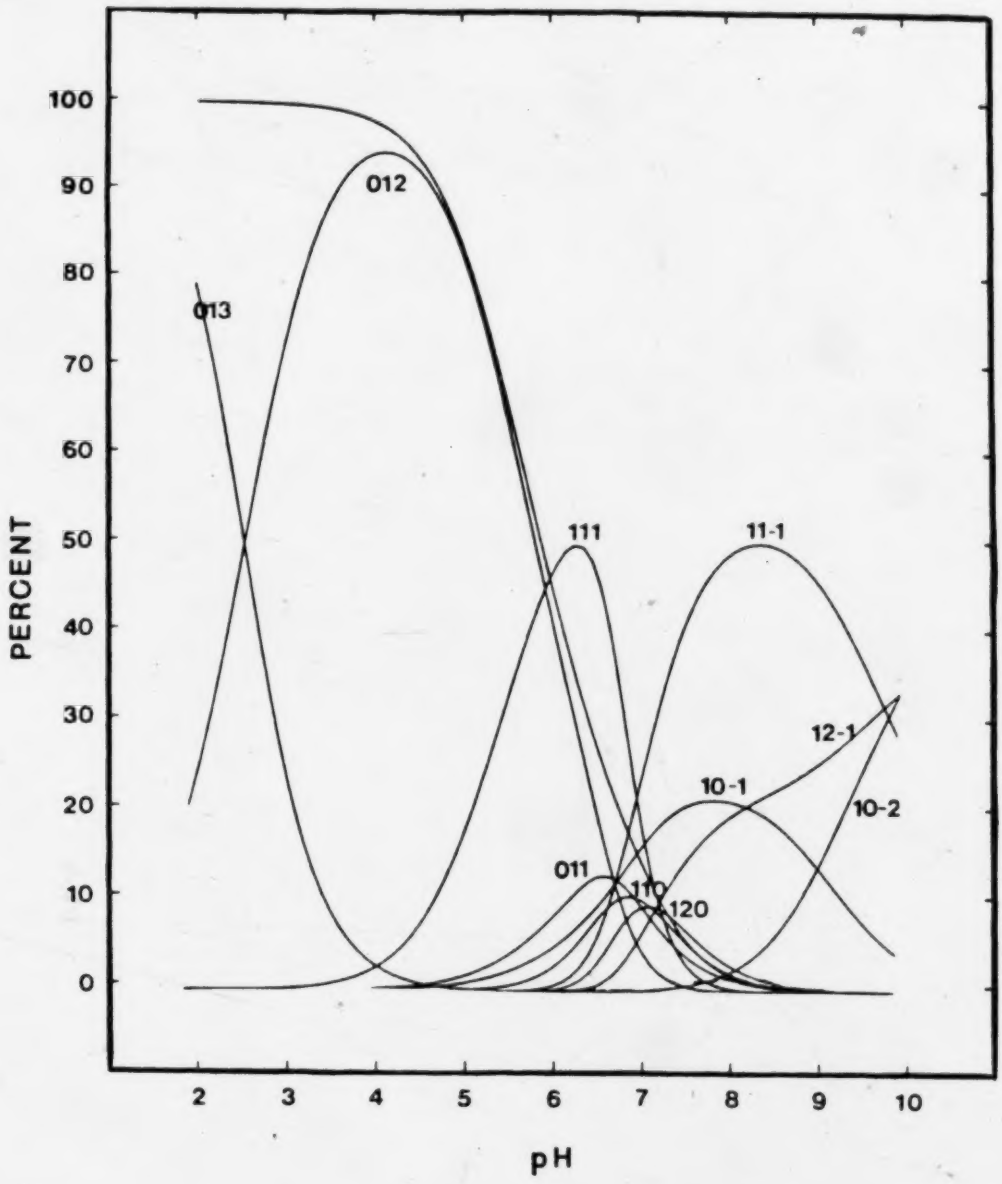


Figure 18. Theoretical Zn(II)-L-alanyl-L-histidine species distributions calculated by COMIX using formation constants shown in Table 14 for a solution containing 0.005 molal each of L-alanyl-L-histidine and zinc(II), reported as percent total L-alanyl-L-histidine. The curve labels designate the species by the subscripts of their MINIQUAD81 formation constants, defined in the text.

3. Glycyl-L-Histidyl-L-Lysine Complexes

a. The Acid Dissociation Constants of Glycyl-L-Histidyl-L-Lysine

When fully protonated, glycyl-L-histidyl-L-lysine is a tetrabasic acid. One titratable proton resides on the amino group of the glycine residue, one on the amino group of the lysine residue, one on the imidazole ring of the histidine residue, and one on the terminal carboxyl group of the lysine residue.

The acetate salt of glyhislys was used in this study. Glyhislys-acetate dissociates in solution to form protonated glyhislys and acetate anion. Since acetate has no appreciable complexation with zinc ($K_{f1}=10^{1.6}$) in comparison with that between glyhislys and zinc, Zn(II)-acetate complexes were not considered in treating the titration data to obtain formation constants. Also, the chemical shift of the methyl resonance of acetic acid was identical to that of free acetic acid, confirming that there was no complexation of Zn(II) in the presence of glyhislys.

The glyhislys molecule can be described by the following macroscopic acid dissociation equilibria:

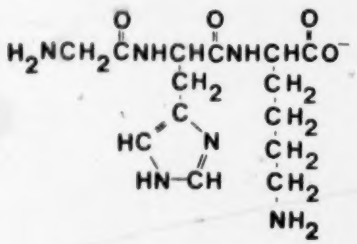
$$AH_4^{3+} \rightleftharpoons AH_3^{2+} + H^+ \qquad (50)$$

$$AH_3^{2+} \rightleftharpoons AH_2^{+} + H^+ \qquad (51)$$

$$AH_2^{+} \rightleftharpoons AH + H^+ \qquad (52)$$

$$AH \rightleftharpoons A^- + H^+ \qquad (53)$$

where 'A' is the fully deprotonated glyhislys molecule shown following.



The acid dissociation constants are defined as follows:

$$K_{a1} = \frac{[H^+][AH_3^{2+}]}{[AH_4^{3+}]} \qquad (54)$$

$$K_{a2} = \frac{[H^+][AH_2^+]}{[AH_3^{2+}]} \qquad (55)$$

$$K_{a3} = \frac{[H^+][AH]}{[AH_2^+]} \qquad (56)$$

$$K_{a4} = \frac{[H^+][A^-]}{[AH]} \qquad (57)$$

Again, these constants were determined titrimetrically, but owing to the cost of the ligand, the experiment was scaled down about five fold. A small titration cell, 5 to 20 mL capacity, was built and used for these experiments. It consists of two compartments joined by a porous frit. One compartment held the test solution while the other held a reference solution which, in this case, was solvent. Further details of its design and testing are found in

Chapters II and III of this thesis. The concentration of glyhislys used in these titrations was about 0.014 molal, standardized as will next be described. The KOH titrant concentration was 0.2503 M with an average deviation of 0.0001 M. All solutions were maintained at a temperature of 25° Celsius throughout each titration and each contained 0.30 molal $[K^+]NO_3^-$ and 1 percent D_2O, the latter for consistency with ^1H NMR experiments.

Because the acetate salt of glyhislys was used, the proton dissociation behavior of this molecule in solutions of 0.30 M $[K^+]NO_3^-$ and 1% D_2O needed to be determined. A solution of acetic acid was prepared in solvent, and titrated with KOH. The titration data was processed with ACBA to determine the acetic acid proton dissociation constant, pK_a. A result of 4.509 with a standard deviation of 0.012 was obtained and used as a constant in subsequent calculations.

ACBA was then used to simultaneously determine the concentrations of ligand and acetate in the stock solution, as well as the ionization constants of glyhislys. The results are presented in Table 15. The pKa used for acetate was kept constant at 4.509. Typical titration curves, both experimental and calculated from the final pK_a's, are shown in Figure 19.

It was found that the proton on the amino group of the lysine residue, whose pKa was found to be 10.482 with a standard deviation of 0.008, was not completely titrated

Table 15.

Determination of the Proton Dissociation Constants and Concentration of Glycyl-L-Histidyl-L-Lysine[a]

DATE/FILE	Conc.[b]	pK_{a1}	pK_{a2}	pK_{a3}	pK_{a4}
FEB29003	0.01360	2.926(7)[c]	6.513(4)	7.879(4)	10.482(8)
FEB29005	0.01359	2.80(4)	6.50(4)	7.85(4)	[10.482]
FEB29008	[0.01360][d]	2.79(5)	6.49(5)	7.83(4)	[10.482]
MAR01002	[0.01360]	2.49(2)	6.49(2)	7.86(2)	[10.482]
WT'D AVERAGE[e]	0.01360	2.91(1)	6.51(4)	7.88(1)	10.48(1)

[a]Calculations done using ACBA.

[b]Concentrations are in units of moles per gram solution.

[c]Values in curved brackets are the standard deviations in the least significant digits of the individual results.

[d]Values in square brackets were kept constant during pK_a calculations.

[e]From equations 27 and 28; simple average for concentration result.

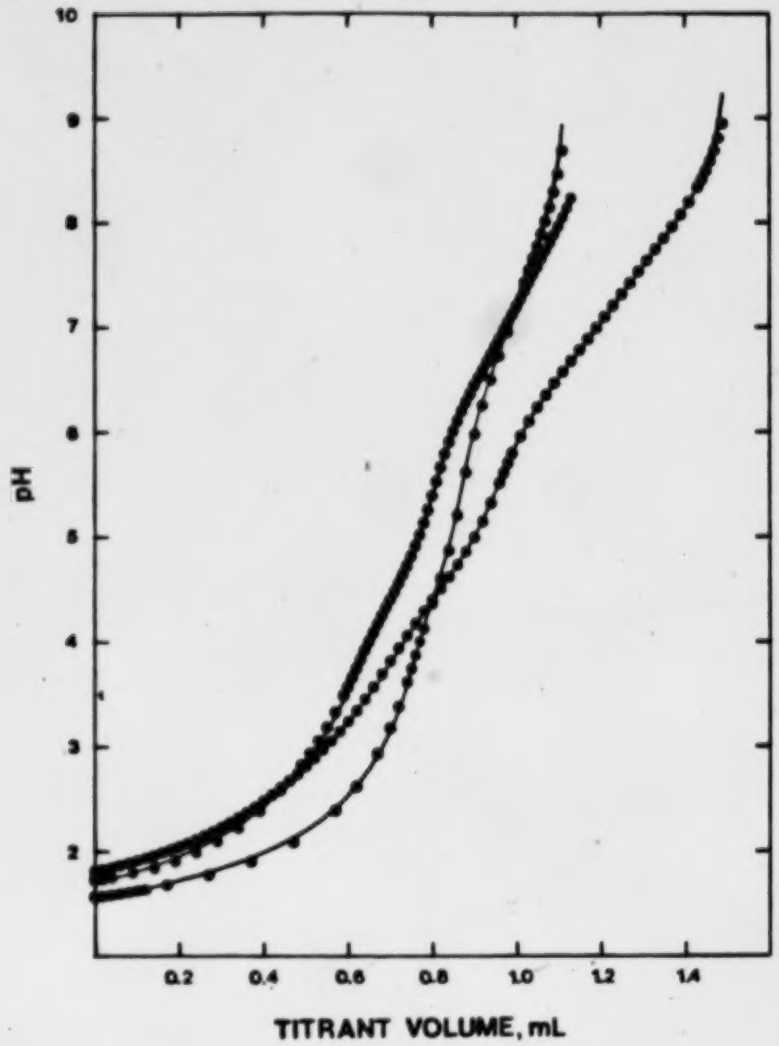


Figure 19. Typical experimental (●) titration curves and corresponding theoretical (—) titration curves calculated using the pK_a's shown in Table 15 for solutions containing 0.008 to 0.014 molal glycyl-L-histidyl-L-lysine. Every second data point is plotted.

until after about pH 11, well past the region of Zn^{2+}-glyhislys complexation. Thus, it was considered to be a non-titratable proton and its dissociation equilibrium was eliminated from the chemical model and its complexation calculations.

This not only aided the calculations by simplifying the chemical model but helped in performing the titrations. The Zn(II)-glyhislys experiment was performed by adding measured amounts of acid-containing metal solution to the titrated ligand solution and then retitrating the combined solution, as has been described in Chapter II. If the ligand was completely titrated, including titration of the lysine residue amino proton, the resultant solution pH was so high that addition of normal amounts of zinc solution and solvent were not enough to lower the pH of the solution to an acceptable level. Adding excess solvent lowered the concentrations of metal and ligand to below mmolal range. Since titration data for the evaluation of Zn(II)-glyhislys formation constants only needed be collected between pH 4 and about 9, and since the lysine proton had a pK_a of 10.482 ±0.008, all ligand titrations after the first one were terminated at about pH 9.

b. The Formation Constants of Glycyl-L-Histidyl-L-Lysine Complexes of Zinc

i. ^1H NMR Experiments

^1H NMR titration experiments were performed as described in Chapter II. The concentrations of metal and ligand were between 0.00179 and 0.00403 molal zinc and between 0.00180 and 0.00523 molal glyhislys. Again, solutions were of 0.300 molal $[K^+]NO_3^-$ and were made 1% in D_2O to provide a lock signal for the spectrometer. The temperature of the spectrometer probe was 25 ±0.1° Celsius.

A behavior similar to that seen in the Zn(II)-glyhis and Zn(II)-alahis systems was also seen in the ^1H NMR spectra of these solutions. The behavior of the imidazole C_2H and C_4H resonances during the titration of ligand solution alone, i.e. the 'free' imidazole resonances, was identical to that seen for glyhis and alahis. Similarily, at about pH 7 and in the presence of zinc, additional resonances were detected in the imidazole region of the spectrum. However, unlike the Zn(II)-glyhis and Zn(II)-alahis situations, two new pairs of resonances were observed for the C_2H and C_4H protons of the imidazole group.

Each resonance of one pair of resonances was located about 0.4 ppm upfield of the 'free' imidazole resonances, just as was the case with the Zn(II)-alahis and Zn(II)-glyhis ^1H NMR spectra. Each resonance of the second set was

located about 0.5 ppm upfield of those of the first set. The chemical shift of the 'free' resonances moved upfield as the pH increased, as expected, but that of both pairs of complexed resonances remained constant over the pH range studied. Typical ^{1}H NMR spectra showing the chemical shift behavior of the imidazole resonances with pH is shown in Figure 20. Graphical representation of the change in chemical shifts of these resonances, 'free' and complexed, with pH is depicted in Figure 21.

The relative areas of the various resonances for 'free' and complexed glyhislys were determined using the cut and weigh method and typical plots of the results are shown in Figure 22. As the pH was increased, the intensity of the 'free' imidazole peaks decreased while that of the complexed imidazole peaks increased. The intensity of the second set of complexed imidazole peaks was always about 30% of that of the first set of complexed imidazole peaks, until about pH 9 when all peaks became too broad to accurately measure. Also, in solutions of a 2:1 metal to ligand ratio, the fraction of the ligand seen to be in the 'free' form never fell significantly below 50%, within the accuracy of the area determination (±10% due to weighing and instrument errors). At pH greater than about pH 10.2, the resonances for complexed glyhislys disappeared and the resonances for 'free' glyhislys reappeared, probably due to hydroxide displacement of the ligand from the metal. Displacement of the ligand by hydroxide ion has been recorded in the

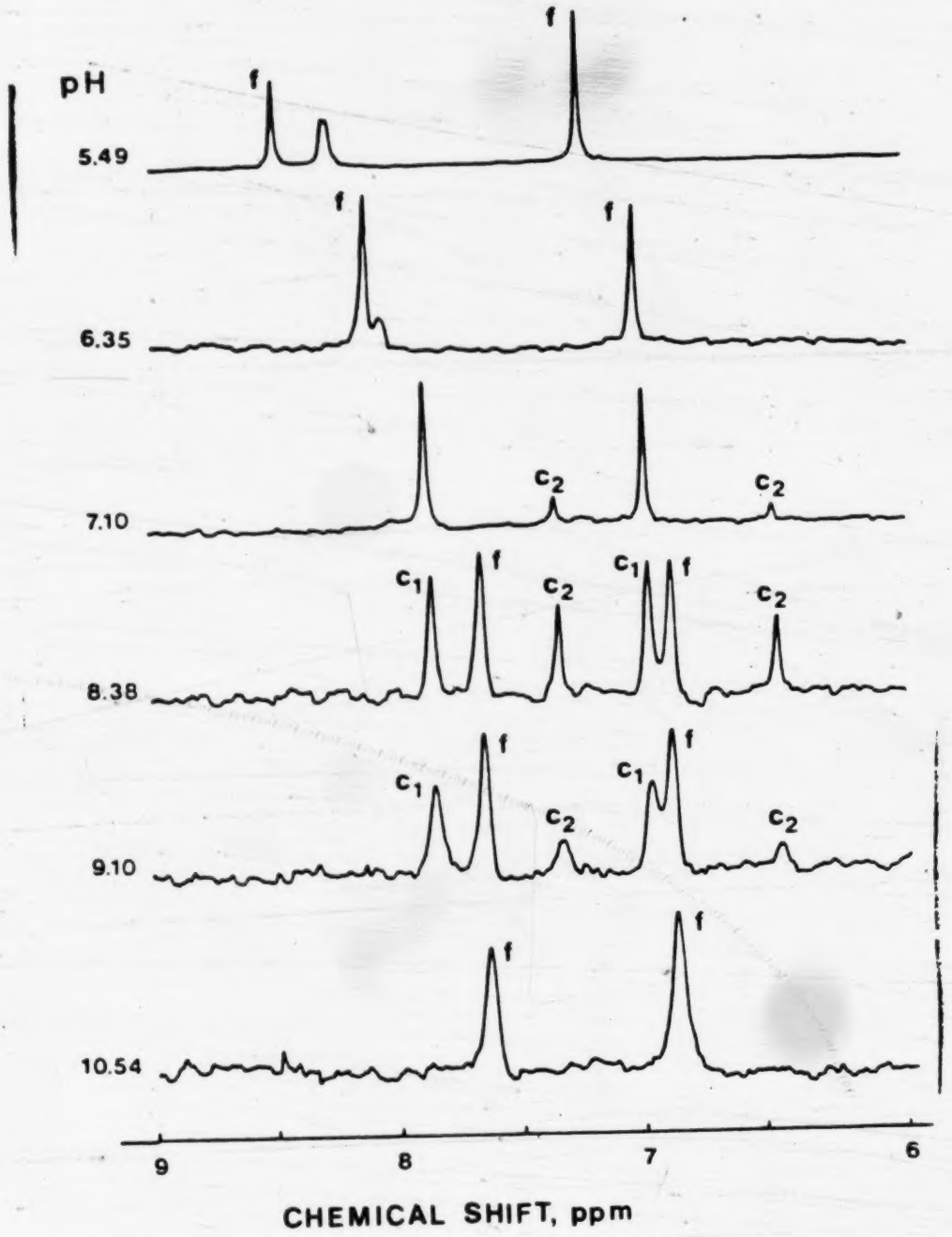


Figure 20. ^{1}H NMR spectra of a solution of 0.005 molal zinc(II) and 0.005 molal glycyl-L-histidyl-L-lysine at several pH values. Resonances labelled 'f' and 'c' are due to 'free' and 'complexed' glycyl-L-histidyl-L-lysine, respectively, defined in the text.

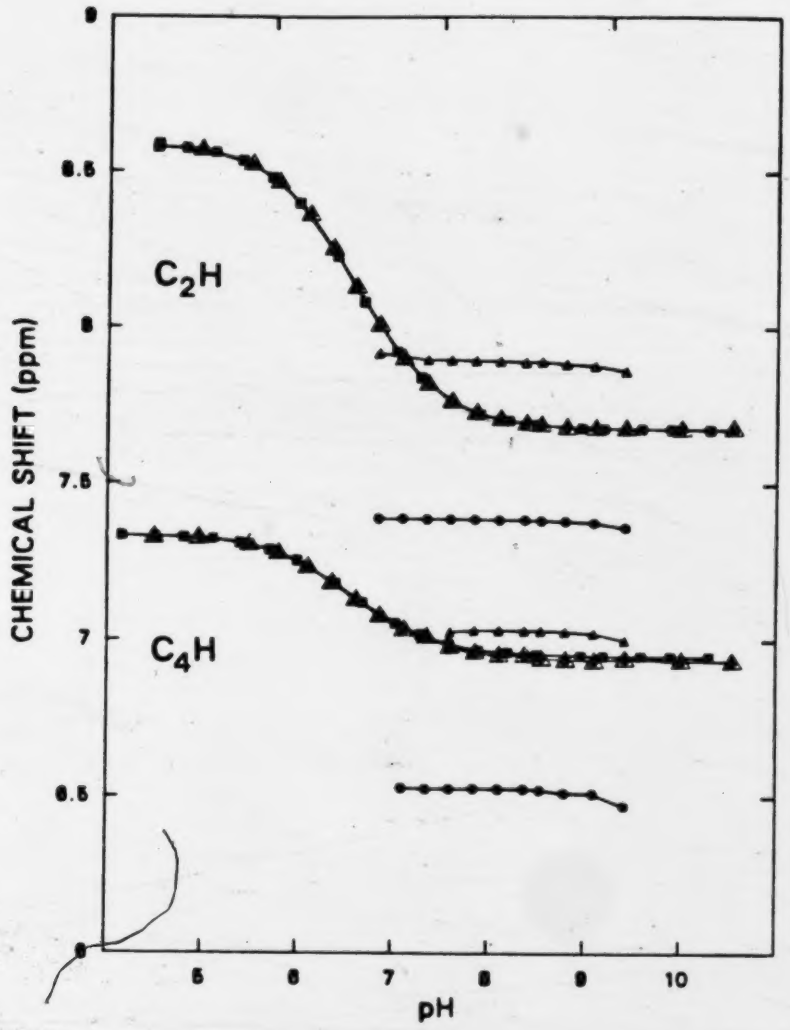


Figure 21. Chemical shift of the imidazole C_2H and C_4H resonances of glycyl-L-histidyl-L-lysine (glyhislys) as a function of pH in a solution of 0.005 molal glyhislys and 0.005 molal zinc(II) compared with those of a solution of glyhislys alone; glyhislys alone(■), complexed glyhislys (●△), and 'free' glyhislys(▲), defined in the text.

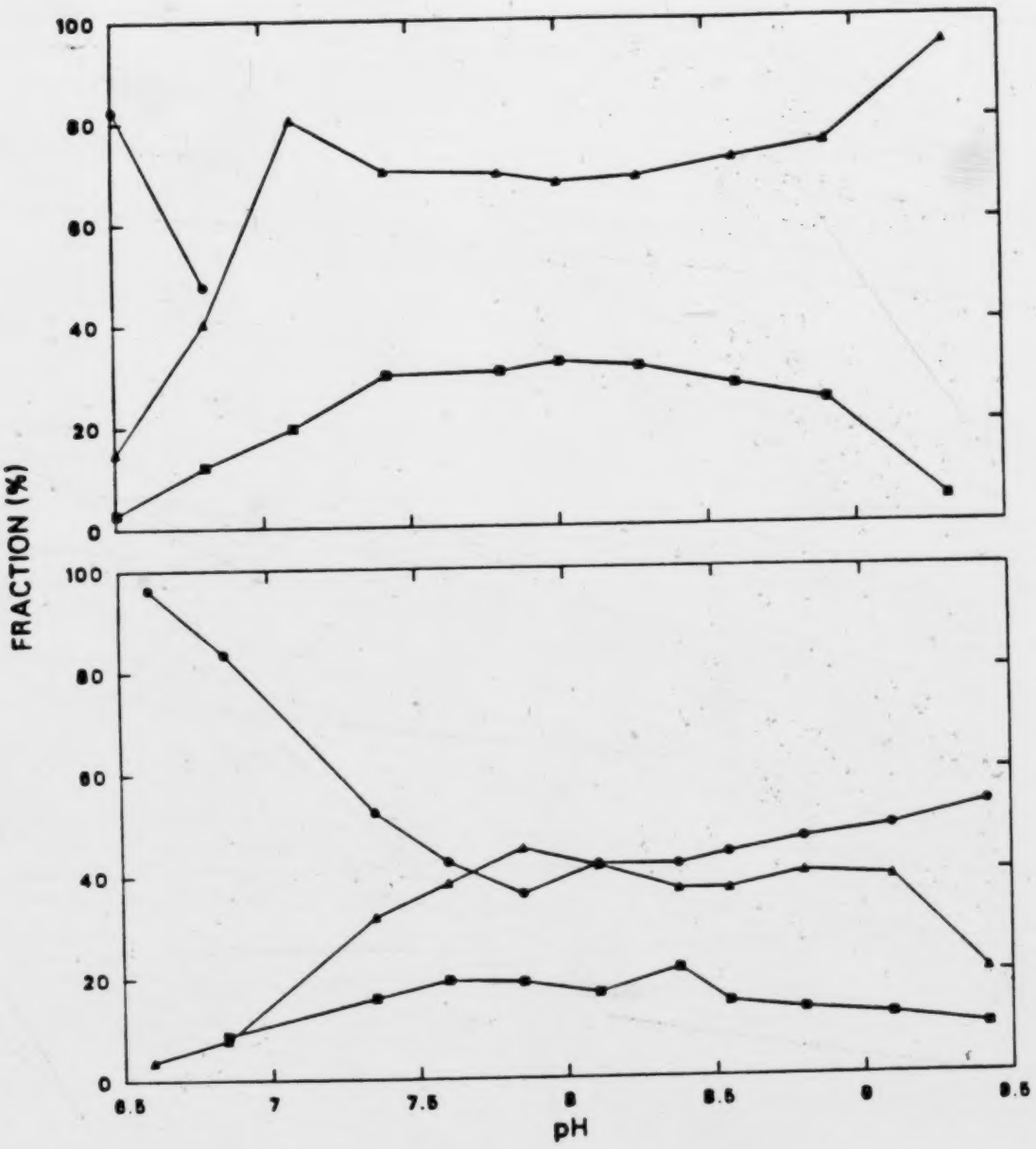


Figure 22. Fraction (%) of the glycyl-L-histidyl-L-lysine (glyhislys) imidazole C_2H resonances in the 'free' (●) and complexed (■△) form as a function of pH for solutions of 0.005 molal glyhislys and 0.005 molal zinc(II) (top) and 0.010 molal glyhislys and 0.005 molal zinc (II) (bottom)

literature for other zinc-ligand systems.

ii. Potentiometric Experiments

On completion of each potentiometric titration of glyhislys and subsequent data processing by the program ACBA, enough standardized zinc solution was added to the titrated ligand solution to reach a predetermined ligand to metal ratio. Solvent solution was also added if necessary to further lower the pH to about 4.0 and to adjust the component concentrations. Efforts were made to closely approximate the concentrations used in the ^1H NMR experiments. Thus, ligand to metal ratios were either 2:1 or 1:1 with the final ligand concentrations being between 0.00192 and 0.00469 molal and metal concentrations of between 0.00189 and 0.00350 molal. All solutions were 0.300 molal in $(K^+)NO_3^-$ and were measured at 25° Celsius. Titration data collected between pH 4.0 and 9.5 (between 200 and -200 mV) was used in determining the Zn(II)-glyhislys formation constants with the program MINIQUAD81.

Table 16 shows the results of the proton balance calculations done using equations 48 and 49, just as were done for the Zn(II)-glyhis and Zn(II)-alahis systems. Again, the basis for these calculations was that the mmoles

Table 16.

Proton Balance Calculations for the

Zn(II)-Glycyl-Histidyl-L-Lysine Titrations

DATE/FILE	Ratio[a]	TITRATABLE[b] MMOLES H^+	MMOLES H^+ TITRATED	DIFFERENCE	MMOLES Zn^{2+}	DIFFERENCE/ MMOLES Zn^{2+}
FEB29004	4.4:2.4	0.6622	0.6951	0.0329	0.0399	0.825
FEB29006	3.7:3.6	0.4343	0.4794	0.0451	0.0425	1.062
FEB29009	2.0:1.9	0.6895	0.7249	0.0355	0.0326	1.089
MAR01003	4.7:2.4	0.4384	0.4624	0.0240	0.0269	0.894
MAR02003	4.7:2.5	0.2403	0.2515	0.0112	0.0132	0.847
MAR08001	1.9:1.9	0.2619	0.2743	0.0124	0.0116	1.067

[a]Ratio of the mmolal concentrations of ligand and metal.

[b]From equation 48.

[c]From equation 49.

of base used in a titration should be the same as the mmoles of ligand times the number of titratable protons per ligand molecule, which, as was discussed, is three for glyhislys.

Relevant zinc(II)-glyhislys complexes were determined by running MINIQUAD81 with the titration data in model select mode as has been described previously. Species thought to be relevant in the Zn(II)-glyhis and Zn(II)-alahis systems were also found to be important in the Zn(II)-glyhislys system, along with a $Zn(glyhislys)(OH)_2$ or $Zn(glyhislysH_{-1})OH$, and a $Zn(glyhislys)_2H_2$ species. The hydroxide species $Zn(OH)^+$ and $Zn(OH)_2$ were also included in the model, using the values of 6.62(1) and 11.44(1) determined earlier as their MINIQUAD81 formation constants.

Values for each of the formation constants were obtained, but species Zn(glyhislys) and $Zn(glyhislys)_2$ were often rejected by MINIQUAD81, making their validity highly questionable. The averages of the rough values for the formation constants of these species obtained in the model select runs were used in the program COMIX to determine their approximate relative distributions over the pH range studied. Any species not forming at least 5% of the total complement of species at some pH was rejected from the model.

Of the above, Zn(glyhislys) and Zn(glyhislysH) were to be eliminated by this test, but were retained because although they may not be very abundant in the system, they were thought to be necessary to complete the system model.

The species $Zn(glyhislysH)_2$, thought to be a minor solution component, never reached greater than 2.0% of the total solution composition at any pH and was rejected from further consideration. The species $Zn(glyhislys)(glyhislysH_{-1})$ showed a maximum abundance of just over 5 percent and so was retained but it was viewed with some scepticism. Therefore, the species found to be important were $Zn(glyhislys)(glyhislysH_{-1})$, $Zn(glyhislysH_{-1})$, $Zn(glyhislys)$, $Zn(glyhislysH)$, $Zn(glyhislysH_{-1})OH$, and $Zn(glyhislys)_2$. Again, the species $Zn(glyhislys)(glyhislysH_{-1})$, $Zn(glyhislys)$, and $Zn(glyhislysH)$ were included to maximize the fit of the data to the theoretical titration curves and to complete the species distributions. Though they were not abundant by the COMIX calculations, they were necessary for a good fit. However, due to their low abundance, only an approximate value for the formation constant was obtained as is indicated by the range of values listed in Table 17.

MINIQUAD81 was then rerun with only these species to obtain the best constants possible for this chemical model and the data collected. Table 17 shows the final results and Figure 23 shows the experimental titration curves and corresponding curves predicted by the above constants for one 1:1 data set and one 2:1 data set. Typical species distributions for the 1:1 metal:ligand system are shown in Figure 24.

Table 17.

Zn(II)-Glycyl-L-Histidyl-L-Lysine Formation Constants

Determined by Potentiometric Titration

DATA/FILE	L:M Ratio[a]	Sum.Sq.[b]	$\log\beta_{11-2}$[c]	$\log\beta_{11-1}$	$\log\beta_{110}$	$\log\beta_{111}$	$\log\beta_{12-1}$	$\log\beta_{120}$
FEB29004	.004:.002	0.008	-11.88(2)[d]	-2.277(7)	---------	9.70(7)	-0.32(7)	--------
FEB29006	.0037:.0036	0.025	-11.59(3)	-2.34(1)	3.1(1)	9.79(8)	---------	7.49(6)
FEB29009	.002:.002	0.107	-11.94(9)	-2.56(3)	---------	--------	0.7(2)	--------
MAR01003	.005:.0024	0.139	-12.87(8)	-2.58(1)	---------	9.22(2)	---------	7.46(4)
MAR02003	.005:.0025	2.55	-9.9(4)[e]	-2.0(1)	---------	11.4(2)[e]	2.1(5)	8.83(9)
MAR08001	.002:.002	6.7	-11.1(5)	-2.15(5)	---------	10.6(1.0)	1.7(2.4)	--------
WT'D MEANS[f]	------	-----	-11.8(1)	-2.3(1)	3.1(1)	9.3(1)	0.5(1)	8.0(1)

[a]Molal ratio of ligand (L) to metal (M).

[b]10^{8} x Sum of squares of the residuals, defined Chapter II.

[c]Symbols defined in section IV B 1 b i.

[d]Numbers in brackets are the std. dev. in the least sig. figs.

[e]Value rejected by the Q test (68).

[f]From equations 27 and 28.

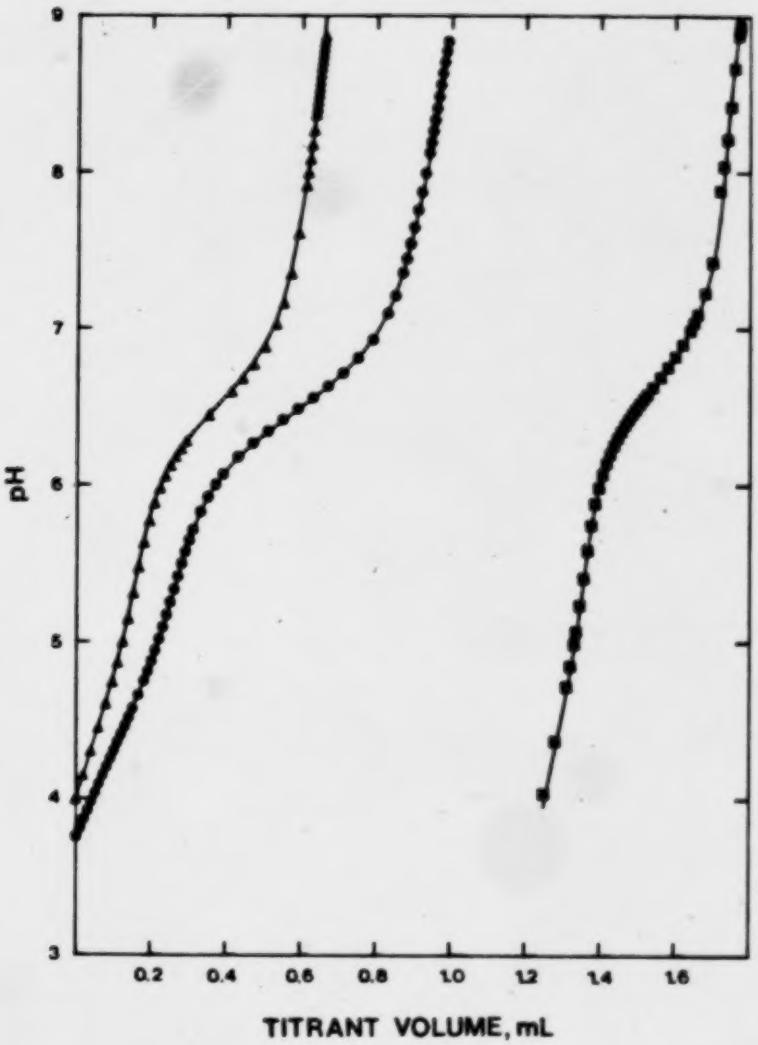


Figure 23. Typical experimental (▲●■) titration curves and corresponding theoretical (—) titration curves calculated from the formation constants shown in Table 17 for solutions containing glycyl-L-histidyl-L-lysine and zinc(II) in ratios of (left to right) 2:1, 2:1 and 1:1. Each solution contained a different amount of free acid, every second data point is plotted.

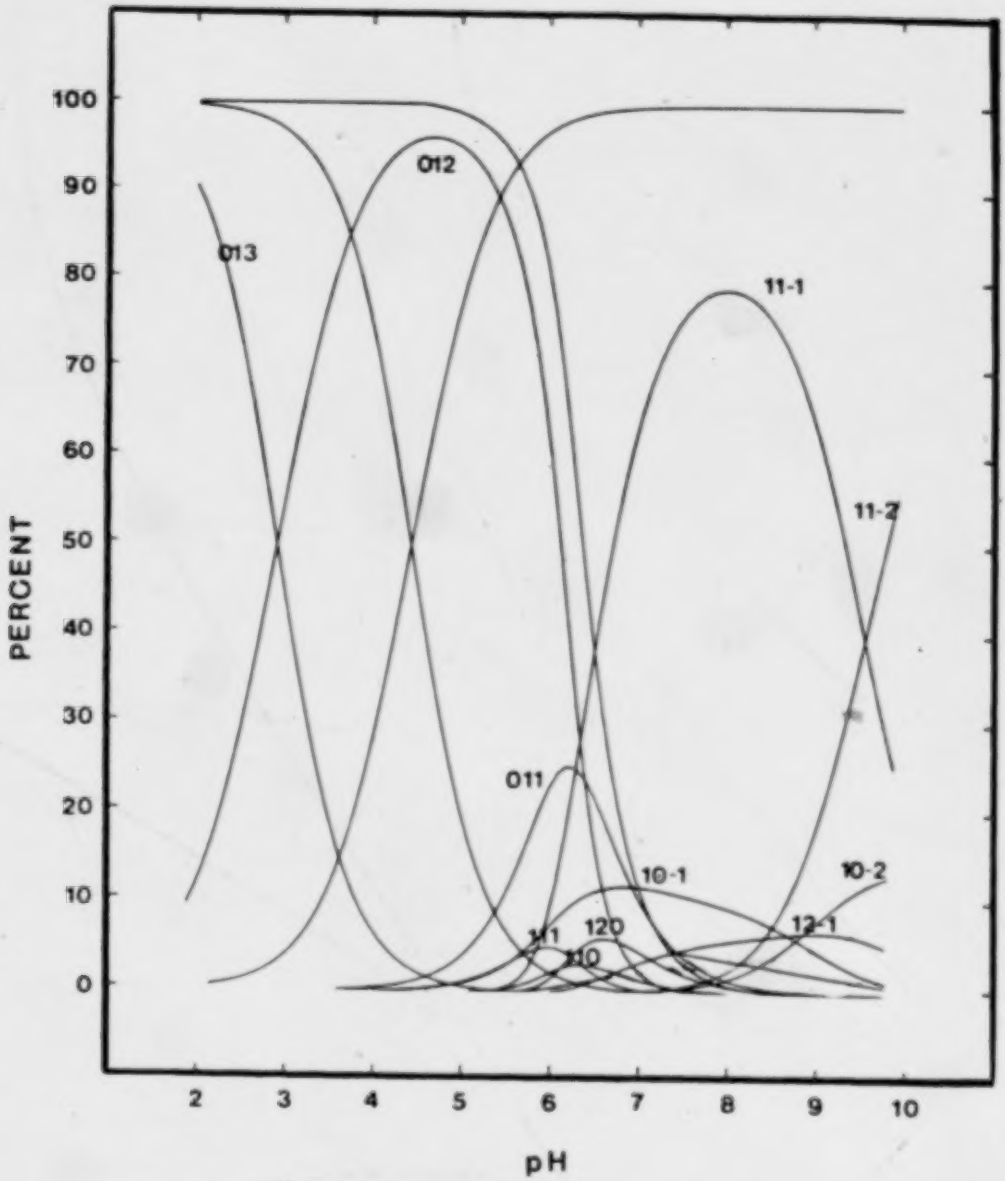


Figure 24. Theoretical Zn(II)-glycyl-L-histidyl-L-lysine (Zn(II)-glyhislys) species distributions calculated using the foramtion constants shown in Table 17 for a solution containing 0.005 molal each of glyhislys and zinc(II), reported as percent total glyhislys. The curve labels identify species by the subscripts of their MINIQUAD81 formation constants, defined in the text.

c. Comparison of ^1H NMR and Potentiometric Results

The Zn(II)-glyhislys formation constants determined in the previous section were used in the program COMIX to obtain theoretical relative distributions of the free and complexed ligand as a function of pH. Free and complexed are defined in the same way as they were defined for the Zn(II)-glyhis and Zn(II)-alahis systems.

Comparison of the ^1H NMR and potentiometric results was not as straight forward as it was for the Zn(II)-glyhis complex system because there are two additional complex resonances and there appeared to be some exchange between the two new complex types, indicated by broadening and distortion of these signals. The results are shown in Tables 1^p and 1^o and will be discussed in a later section. In Table 1^o, where the solutions studied contained zinc and ligand in a 1:1 ratio, the free ligand resonances completely disappeared on reaching pH 7.

Table 18.

Comparison of Observed[a] and Predicted[b] Percentages of Free[c] Glyhislys in Solutions Containing Glyhislys and Zinc(II)[d]

pH	CASE 1 Observed	CASE 1 Predicted	CASE 2 Observed	CASE 2 Predicted
6.50	85	78	82	71
6.65	71	69	----------	
6.81	68	58	48	51
7.00[e]	22	46	----------	

[a] From ^{1}H NMR experiments.
[b] From COMIX treatment of formation constants shown in Table 17.
[c] Defined in text.
[d] Equimolal metal and ligand; CASE 1, 0.002 molal each: CASE 2, 0.004 molal each.
[e] No free ligand determined in NMR spectra above this pH.

Table 19.

Comparison of Observed and Predicted Percentages of Free Glyhislys in Solutions Containing Glyhislys and Zinc(II)[a]

pH	CASE 1 Observed	CASE 1 Predicted	CASE 2 Observed	CASE 2 Predicted
6.50	92	83	----------	
6.60	----------		96	78
6.65	79	77	----------	
6.79	75	73	----------	
6.85	----------		84	72
6.95	71	69	----------	
7.35	----------		52	63
7.51	46	60	----------	
7.60	----------		43	60
7.85	----------		36	57
8.10	----------		42	55
8.20	44	54	----------	
8.35	----------		42	53
8.55	----------		44	52
8.65	47	52	----------	
8.80	----------		47	52
9.10	----------		49	51
9.40	63	51	53	51

[a]Definitions as for Table 17. Solution glyhislys:Zn(II) concentrations as follows; CASE 1, 0.004:0.002 molal: CASE 2, 0.005:0.0025 molal.

C. Discussion

The purposes of the experiments described in this chapter were (a) to determine if zinc(II) binds strongly to small peptides in which histidine is the second amino acid from the N-terminal end and (b) to try to elucidate the nature of the complexes which do form. It has been reported (15,32-34) that zinc(II) binds to glycyl-L-histidine with a proposed ligand deprotonation at the amide nitrogen, as has been established for several other metals with this ligand (16-31). By comparing the results of ^{1}H NMR and potentiometric experiments, it was hoped that this relationship between zinc(II) and small histidine-containing peptides, which might model the N-terminal end of the beta chain of the hemoglobin molecule, could be further verified.

Proton balance calculations indicate that an extra proton is being titrated per glyhis for each zinc(II) ion present. In solutions of less than 1:1 mole ratio of glyhis to zinc(II), the fraction of excess protons titrated from the complex is between 0.78 and 0.93, which corresponds to the titration of an additional proton from the ligand per zinc(II) atom present. The fact that the fraction of excess protons is less than 95% of the total ligand present might be due to the formation of zinc hydroxides before complete zinc(II)-ligand complexation can take place. The results of these calculations are consistent with the hypothesis that the amide proton, not normally titratable under the

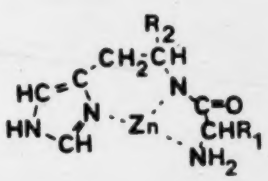
conditions of the experiment, is being released on binding to zinc(II).

During the potentiometric and ^1H NMR titrations, precipitate is seen above pH 8.5 in solutions containing a 1:1 mole ratio of glyhis or alahis to zinc(II), indicating that some form of zinc hydroxide or another insoluble zinc complex is being formed. Also, the same precipitate is seen in any Zn(II)-ligand solution of mole ratio less than 1:1 but not in those when the mole ratio is even slightly above 1:1. The same is observed in potentiometric titrations. Either the mole ratio is actually somewhat less than 1:1 and free zinc(II) is forming its hydroxide or hydroxide is being incorporated into the Zn(II)-glyhis or Zn(II)-alahis complex. The latter would not likely be insoluble since it is doubtful that the complex could achieve electroneutrality at a pH above 8.5 where the precipitate is seen.

Because the above precipitate appears in solutions at a fairly low pH, COMIX-calculated species distributions for the Zn(II)-ligand systems were recalculated using published $Zn(OH)_x$ constants (64), and the species $Zn(OH)^+$ and $Zn(OH)_2$ were found to be abundant over the pH range of interest. These formation constants were then determined in our laboratory for the ionic conditions of the Zn(II)-ligand experiments. They compared well with those of the literature and were used in further calculations. Their inclusion in the chemical model for the system improved the fits of the data to the theoretical curves, sometimes by as

much as a factor of ten in the sum of squares of the residuals.

The chemical models determined by the potentiometric titration results are shown in Figures 25 to 27. Structure IV illustrates the proposed nature of the binding of Zn(II) to these ligands in the complexes where the amide nitrogen is deprotonated, i.e. in those complexes having formation constant designations β_{11-1}.



IV

In this figure, R_1 is a proton for the molecules Zn(II)-glyhis and Zn(II)-glyhislys and CH_3 for Zn(II)-alahis. Similarly, R_2 is the carboxylate group, $-COO^-$ for the molecules Zn(II)-glyhis and Zn(II)-alahis and is the lysine residue($-CONHCH((CH_2)_4NH_3^+)COO^-$) in the molecule Zn(II)-glyhislys. It was determined that this proposed structure is feasible, since these complexes can be constructed with space-filling molecular models.

Neither the results of the potentiometric experiments nor the ^1H NMR experiments can exclude the existence of 2:1 complexes, though such complexes where both ligands have had an excess proton, i.e. the amide proton, titrated from them is extremely unlikely. Indeed, the results of the

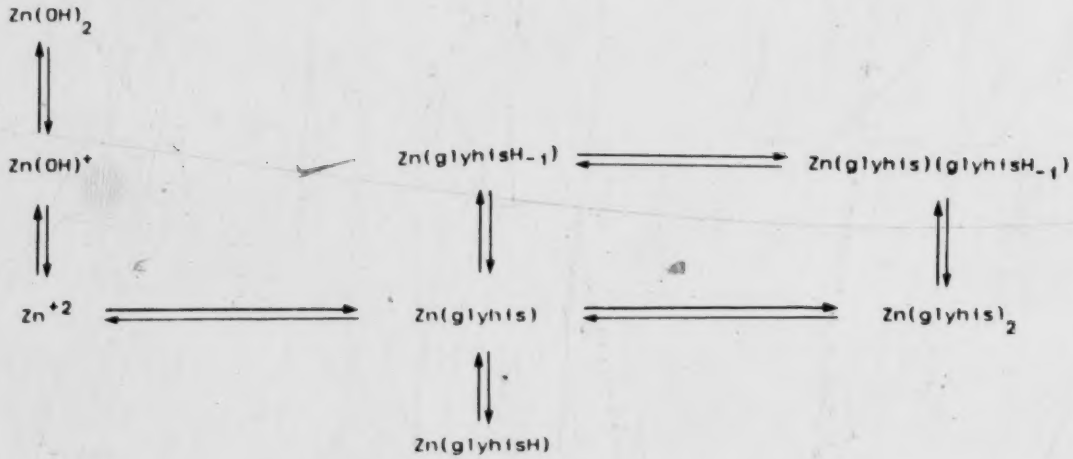


Figure 25. Proposed Zn(II)-glycyl-L-histidine association scheme.

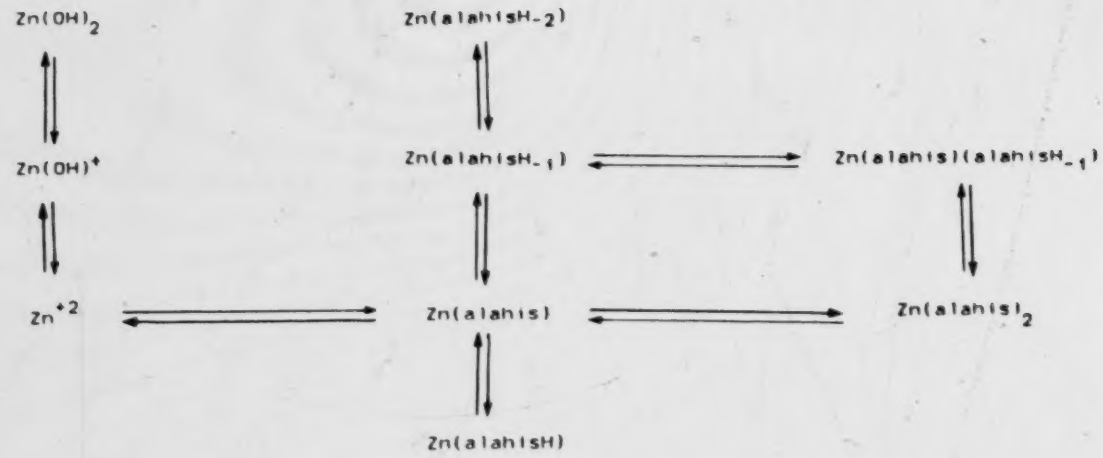


Figure 26. Proposed Zn(II)-L-alanyl-L-histidine association scheme.

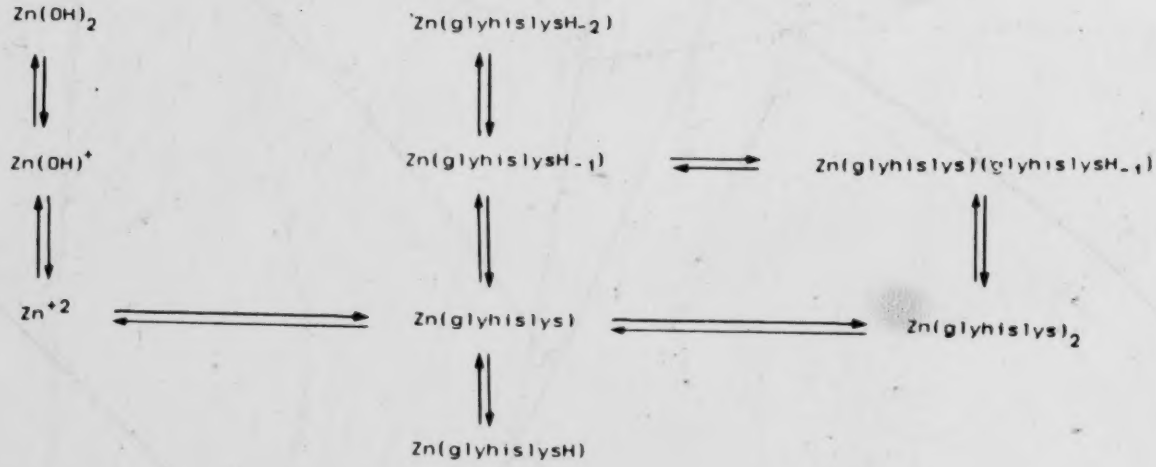


Figure 27. Proposed Zn(II)-glycyl-L-histidyl-L-lysine association scheme.

potentiometric experiments indicate a definite presence of ?:1 complexes, especially solutions containing at least twice as much ligand as metal. Still the proton balance calculations show that only one of the ligands may be amide deprotonated on binding to zinc(II). Even with the ligand glyhislys, where the possibility exists of titrating two amide protons on a single molecule, only one additional proton is titrated. This is further substantiated by the ^1H NMR titration results in that solutions containing twice as much ligand as metal never show significantly more than half of the imidazole resonances in the complexed, slowly exchanging form. Thus, there is probably little ?:1 complex of this nature being formed.

This proposed amide deprotonated species seems to be quite strong. In the ^1H NMR experiments with solutions of equimolal metal and ligand, increases in pH above about 7 resulted in the majority of the ligand being in the kinetically stable complexed forms. Also, the formation constants β_{11-1} and β_{12-1} are quite high with log values of about -?.1 and 1.4 respectively, irrespective of the ligand, and COMIX-calculated species distributions show that the species with formation constant β_{11-1} is especially predominant in solution over the pH range studied.

It has been suggested (63) that inclusion of a species of the type $Zn(glyhisH_{-1})OH$ or $Zn(glyhisH_{-2})$ is necessary to completely describe the Zn(II)-glyhis system. Our analyses show that the abundance of this species over the course of a

normal titration (pH 4 to 9.5) does not exceed 5 percent of the total species distribution and so may be considered nonessential. The same is true for the Zn(II)-alahis system, but the abundance of this species type in the Zn(II)-glyhislys system is sufficient to merit its consideration.

Consider, now, more results of the ^1H NMR studies. In all instances, the chemical shifts of the free imidazole resonances of ligands in solutions containing zinc(II) are somewhat upfield of those in solutions of ligand alone at the same pH. This indicates the presence of some Zn(II)-ligand complexation. The fact that the chemical shift of these resonances further changes with pH indicates that there exists a fast exchange of ligand on the metal with that in solution. However, the appearance of the additional resonances in the imidazole region indicate that in the pH range 4 to 9 complexes form which are kinetically stable on the NMR time scale, i.e. the ligand is not exchanging between the free and complexed forms. The chemical shifts of the complexed imidazole resonances do not change appreciably with pH for these complexes. This is consistent with the proposed structure involving deprotonation of the amide nitrogen with complexation of zinc(II) at that site in addition to binding at the glycine amino and imidazole N-1 nitrogen. A five or six membered ring is formed when zinc(II) binds to the above sites, and such rings are known

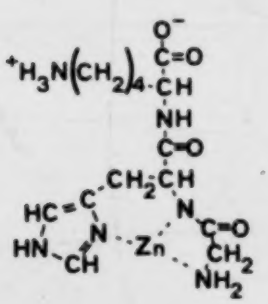
to be fairly stable structures.

In the case of glyhislys, there were not just two additional imidazole resonances observed but four, corresponding to two additional C_2H and two additional C_4H resonances, which suggests the formation of two unique new complexes. The fact that the relative intensities of the two sets of additional peaks remained fairly constant with changes in pH indicates that the second set of resonances are due to a complex different from the type described by Structure IV and not simply a species formed from the first complex by titration of another amide proton or addition of a hydroxide ion. Also, considering the absence of pH dependence of the chemical shifts of the second set of complexed imidazole resonances, the binding of the second group is probably fairly strong, again, possibly involving deprotonation at the lysine amide nitrogen.

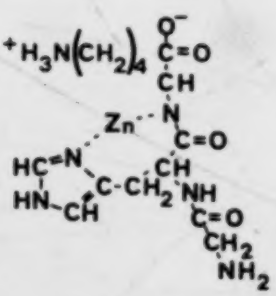
The Zn(II)-glyhislys complexes were initially thought to be much stronger than those of glyhis or alahis, since there was a total absence of free C_2H and C_4H resonances after reaching a pH of about 7 in titrations of solutions of a ligand to metal ratio of 1 to 1. However, the formation constants for species thought to be amide deprotonated, i.e. those having formation constants β_{11-1} and β_{12-1} and thought to give rise to the complexed imidazole resonances, were determined by potentiometric titration and did not differ greatly from ligand to ligand. From this, it was later surmised that the absence of the free imidazole resonances

at pH's above 7 resulted from the formation of two different amide deprotonated Zn(II)-glyhislys complexes and that exchange between them and the free ligand caused broadening and the disappearance of the 'free' resonances.

In formulating structures for these species, the following was considered. Throughout the course of the Zn(II)-glyhislys ^{1}H NMR titration, the ratio of the intensities of the two sets of complexed imidazole resonances is a fairly constant 3:1, suggesting that the complex of the first type is somewhat more thermodynamically stable than the second. Assuming that the first type of complex is that involving deprotonation of the amide nitrogen of the histidine residue, as has been suggested and will further be discussed, one might consider the second type of complex to involve a similar deprotonation of the amide nitrogen of the lysine residue to form a 7 membered ring with the metal and imidazole group. Structures V and VI are possible representations for these complexes.



V



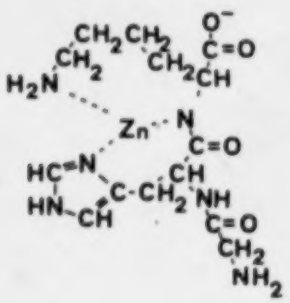
VI

This larger ring would be somewhat less thermodynamically stable than the proposed 6 membered ring involving complexation at the glycine side of the histidine residue. Therefore, this complex would be expected to be less abundant than the other and would explain the 3:1 ratio observed.

There seems to be some exchange between the two zinc(II)-glyhislys complexes since, as the pH is raised, the complex resonances distort and broaden. Perhaps there is exchange between structures V and VI, or these structures may also exchange between being amide nitrogen deprotonated and being without amide deprotonation.

It might also be considered that there may be some other donor group involvement to form this stable, fairly non-exchanging complex. The structure of such a complex might be like that of V or VI but with the lysine amino or its carboxyl group bound to the metal. Using molecular models, a molecule with structure VII was built. Also, a structure of this sort having the lysine amide nitrogen deprotonated instead of the histidine amide nitrogen was found to be possible using molecular models. The fact that the lysine amino proton, having a pK_a of greater than 10, would first have to be titrated from the residue for the complexation to occur in this manner would tend to disprove the existence of this complex in the pH range studied. Although it has been long established that many metal ions,

including Zn(II), have the effect of reducing the pK_a's of ligands in their presence it is unlikely that the lysine amino pK_a could be reduced to a value value low enough to allow the formation of Structure VII at a pH of about 7.



VII

Other structures were also built where there was binding by the lysine carboxyl group but they were too strained to be realistic. Similarily, a structure like VI but where the glycine amino nitrogen was bound was also strained.

In conclusion, it would seem that zinc(II) forms quite strong complexes with histidine-containing peptide ligands having amino acid sequences similar to that found at the N-terminal end of the beta chain of hemoglobin. The strength of this complexation may lie in the binding of the amide nitrogen to the metal with deprotonation at that nitrogen to form 6 and 7 membered rings as in structures IV to VI. If

the ligands used here truly model those of the N-terminal end of the beta chain of hemoglobin, then the results of this study suggest that the N-terminal end of the beta chain could be the site of the binding of zinc(II) to hemoglobin.

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Appendix

```
10 '*************************************************************************
20 '*                                                                       *
30 '*           TITRATE.BAS by A.Arnold & S.Daignault                       *
40 '*                Department of Chemistry                                *
50 '*                 University of Alberta                                 *
60 '*               Edmonton, Alberta T6G 2G2 Canada                        *
70 '*                                                                       *
80 '*************************************************************************
90 '
100 CLEAR                       'clear the data space
110 POKE &H5B,1                 'set top of screen scroll to line 1
120 DEFDBL A-H,O-Z              'all non-integers are double precision
130 PPAGE%=0                    'parameters are on page 0
140 DPAGE%=1                    'data is on page 1
150 TPAGE%=2                    'ph meter test data is on page 2
160 DISK$="a:"                  'initialise working drive to a: for error checkin
170 SCREEN 0,0,PPAGE%,PPAGE%    'set up parameter screen 0
180 VPAGE%=PPAGE%:APAGE%=PPAGE% 'keep track of virtual and active pages
190 ON ERROR GOTO 4330          'error trapping routine
200 FALSE=0:TRUE=NOT FALSE      'define true and false
210 TEST=FALSE                  'initialise the test flag
215 SPIKE=FALSE                 'initialise the spike flag
220 CLS:KEY OFF
230 DIM PH(1000),VOL(1000)      'set up arrays for siniquad81 data presentation
240 DIM X(300),BETA(20),JPOT(20),JQR1(20,5),IKEY(20),TOTC(5),ADDC1(5)
250 DISK$="b:"                  'change working drive to b:
260 ENDVOL=1!                   'maximum volume delivered by the burette
270 DEF FNBCONV%(X)=X-6*(X\16)'converts decimal to BCD
280 DEF FNTIME(I$)=3600*VAL(LEFT$(I$,2))+60*VAL(MID$(I$,4,2))+VAL(RIGHT$(I$,2))
     'converts time to seconds
290 REM ************** insert all introductory text here ******************
300 PRINT "                     IBM PC Titration Program"
310 PRINT "                      A.Arnold & S.Daignault"
320 PRINT
330 PRINT "                    (last revision Oct.3,1984)"
340 PRINT:PRINT:PRINT
350 PRINT "This program is for use with an Orion 701A pH meter."
352 METER$="o"
353 REM the following few lines were useful when the program was run with
354 REM either the fisher 520 or orion 701a ph meter and the meter used was
355 REM at the operator's discretion.  these lines are kept here in case the
356 REM titration system is restored to that state.
360 REM INPUT "Will a Fisher or Orion pH meter be used (f/o)";METER$
361 REM METER$=MID$(METER$,1,1)
362 REM IF METER$="F" THEN METER$="f"
363 REM IF METER$="O" THEN METER$="o"
364 REM IF METER$="f" OR METER$="o" THEN GOTO 370 ELSE LOCATE CSRLIN-1,POS(0)
365 REM SOUND 300,5:GOTO 360
370 DREADY=&H40             'data ready for Orion 701A
375 IF METER$="o" THEN MASK=&H14 ELSE MASK=&H5   'masks for mV/pH meter modes
380 IF METER$="o" THEN PLUSMV=&H14 ELSE PLUSMV=&H0  '+mV indicator
382 IF METER$="o" THEN MINUSMV=&H4 ELSE MINUSMV=&H4  '-mV indicator
384 IF METER$="o" THEN PLUSPH=&H10 ELSE PLUSPH=&H1  '+pH indicator
390 IF METER$="o" THEN ONEK=&H1 ELSE ONEK=&H40     'MSD indicator
410 IF METER$="o" THEN NTIMES%=30 ELSE NTIMES%=100  'number of times to read the
 meter
420 PRINT:PRINT "While running this program, the operation of the optoisolator a
nd pH-meter can  be checked by pressing F5."
430 PRINT:PRINT "Please turn up the sound to a reasonable level, because the pro
gram "
440 PRINT "will politely 'beep' at you if you make a mistake!"
450 PRINT "And please take note of any malfunctions and report them to us."
460 PRINT
470 REM ****************** end of introductory text ********************
480 PRINT:PRINT "All data and parameters will be stored on disk ";DISK$
```

```
490 PRINT:INPUT "Do you want a hard copy of this titration? (y/n)";HARD$
500 HARD$=MID$(HARD$,1,1)    'hardcopy flag
502 IF HARD$="Y" THEN HARD$="y"
504 IF HARD$="N" THEN HARD$="n"
510 IF HARD$="n" OR HARD$="y" THEN GOTO 530 ELSE LOCATE CSRLIN-2,POS(0)
520 SOUND 300,5: GOTO 490
530 CLS
540 PRINT CHR$(201)+STRING$(77,205)+CHR$(187)
550 PRINT CHR$(186)+SPACE$(77)+CHR$(186): PRINT CHR$(186);
560 IF HARD$="n" THEN GOTO 580
570 LPRINT STRING$(79,95)    'print a line across the page
580 COLOR 15,0
590 IF METER$="o" THEN PRINT "        IBM PC /Orion Titration Program      ";
    ELSE PRINT "        IBM PC/Fisher Titration Program      ";
600 IF HARD$="n" THEN GOTO 650
610 LPRINT CHR$(27);"E"   'turn on emphasized print
620 IF METER$="o" THEN LPRINT "        IBM PC /Orion Titration Program      ";
    ELSE LPRINT "        IBM PC/Fisher Titration Program      ";
630 LPRINT DATE$;"  ";TIME$
640 LPRINT CHR$(27);"F"   'turn off emphasized print
650 PRINT DATE$;"  ";TIME$;
660 COLOR 7,0
670 PRINT SPACE$(12)+CHR$(186):PRINT CHR$(186)+SPACE$(77)+CHR$(186)
680 PRINT CHR$(200)+STRING$(77,205)+CHR$(188)
690 POKE &H5B,6          sets top of scroll window see PC 2(5) p648
700 COL(PPAGE%)=POS(N):ROW(PPAGE%)=CSRLIN    'store current cursor position
710 SCREEN 0,0,DPAGE%,PPAGE%    'display key on data page only
720 VPAGE%=PPAGE%:APAGE%=DPAGE%  'keep track of virtual & active pages
730 CLS
740 LOCATE 25,1              'print the key information
750 COLOR 0,7:PRINT " F1 ";:COLOR 7,0:PRINT " Pause   ";
760 COLOR 0,7:PRINT " F2 ";:COLOR 7,0:PRINT " Parameter Revision ";
770 COLOR 0,7:PRINT " F3 ";:COLOR 7,0:PRINT " Stop Titration ";
780 COLOR 0,7:PRINT " F4 ";:COLOR 7,0:PRINT " Screen ";
790 COLOR 0,7:PRINT " F5 ";:COLOR 7,0:PRINT " Test ";
800 SCREEN 0,0,PPAGE%,PPAGE%  :VPAGE%=PPAGE%:APAGE%=PPAGE%
810 LOCATE ROW(PPAGE%),COL(PPAGE%)
930 REM
940 NUMBER%=1              'initialise data counter
950 GOSUB 3310             'enter the titration parameters
960 REM
970 PRINT:INPUT "Will this data be processed with MINIQUAD81, or in another way?
 (m or o)";DPANS$
980 DPANS$=MID$(DPANS$,1,1) 'read the Miniquad flag
990 IF DPANS$="M" THEN DPANS$="m"
1000 IF DPANS$="O" THEN DPANS$="o"
1010 IF DPANS$="m" OR DPANS$="o" THEN 1030 ELSE LOCATE CSRLIN-2,POS(0)
1020 SOUND 300,5 :GOTO 970
1030 IF DPANS$="o" THEN GOTO 1100
1040 IF PAR$="n" THEN GOSUB 4460
1050 IF PAR$="y" AND EOF(1) THEN PRINT "No MINIQUAD parameters in ";PARFILE$ :GO
SUB 4460
1060 IF PAR$="y" AND NOT EOF(1) THEN GOSUB 5260
1070 REM
1080 REM if miniquad is being used, set the tab according to how many reactants
there are
1090 IF DPANS$="M" THEN ITAB=6+((NMBE-1)*10) ELSE ITAB=0
1100 PRINT:INPUT "What is the initial buret volume (usually 0.0000,of course)";V
OL(1)
1110 PRINT
1120 COLOR 31,0
1130 PRINT SPACE$(19)+"MAKE SURE THAT THE 1.000mL BURET IS READY"+SPACE$(19)
1140 IF HARD$="y" THEN PRINT SPACE$(23)+"AND THAT THE PRINTER IS TURNED ON"
1150 COLOR 7,0
1160 PRINT SPACE$(80)
1170 PRINT:PRINT "Press any key to start the titration....."
1180 IF INKEY$="" THEN 1180
1181 REM set up function keys for program interrupts
```

```
1182 ON KEY(1) GOSUB 2950:KEY(1) ON    'pause interrupt
1183 ON KEY(2) GOSUB 3110:KEY(2) ON    'parameter revision interrupt
1184 ON KEY(3) GOSUB 2540:KEY(3) ON    'stop titration
1185 KEY(4) OFF
1186 ON KEY(5) GOSUB 5500:KEY(5) ON    'test pH meter
1187 KEY(6) OFF:KEY(7) OFF:KEY(8) OFF:KEY(9) OFF:KEY(10) OFF
1190 OUT 831,155            'set up control port in mode 0 all 24 bits input
1200 LOCATE 12,1
1210 FOR I%=1 TO 12:PRINT SPACE$(79):NEXT I%
1220 LOCATE 13,1
1230 ON KEY(4) GOSUB 5460:KEY(4) ON   'screen toggle
1240 COL(PPAGE%)=POS(N):ROW(PPAGE%)=CSRLIN     'store current cursor position
1250 SCREEN 0,0,DPAGE%,DPAGE% :VPAGE%=DPAGE%:APAGE%=DPAGE%
1260 LOCATE 1,1
1270 REM program the 8522 parallel ports for data acquisition
1280 REM
1290 IC%=INP(830)             'read port C ,1st digit and meter modes
1300 REM print out the listing headings
1310 PRINT
1320 PRINT"             VOLUME          ";
1330 IF (IC% AND MASK)=PLUSPH THEN PRINT"pH          ";CHR$(235);"(pH)";
   ELSE PRINT"mV          ";CHR$(235);"(mV)";
1340 PRINT "        SLOPE       DELAY"
1350 PRINT"               mL                          /min.                    sec
."
1360 PRINT:PRINT STRING$(79,196)
1370 IF HARD$="n" THEN GOTO 1440
1380 LPRINT:LPRINT
1390 LPRINT"             VOLUME          ";
1400 IF (IC% AND MASK)=PLUSPH THEN LPRINT"pH          ";CHR$(100);"(pH)";
   ELSE LPRINT"mV          ";CHR$(100);"(mV)";
1410 LPRINT"        SLOPE       DELAY"
1420 LPRINT"               mL                          /min.                    se
c."
1430 LPRINT STRING$(79,95)    'print a line across the page
1440 REM
1450 REVISE=FALSE        'reset the revision flag
1460 REM ********************* main program loop ***************************
1470 REM
1480 DELAY=0'              'set time delay to 0 at start of readings
1490 STARTTIME=FNTIME(TIME$)   'store time at start of read loop
1495 SPIKE=FALSE               'reset spurious reading flag
1500 REM read ports A and B NTIMES% times if meter is ready (port C bit 6=1)
1510 IF NOT TEST THEN BEEP
1520 FOR I%=1 TO NTIMES%
1530 IF TEST THEN SOUND 500,1
1540 IC%=INP(830):IF METER$="f" THEN 1560
1550 IF (IC% AND DREADY)<>DREADY THEN 1540
1560 IA%=FNBCONV%(INP(828))    'read port A ,5th and 4th digits
1570 IB%=FNBCONV%(INP(829))    'read port B ,3rd and 2nd digits
1580 IDATA%=IA%*100+IB%        'combine the last four digits
1590 IF (IC% AND ONEK)=ONEK THEN IDATA%=IDATA%+10000 'add MSD if >=1000
1600 IF (IC% AND MASK)=MINUSMV THEN IDATA%=-IDATA%    'negate if <0
1610 IF (IC% AND MASK)=PLUSPH THEN X(I%)=IDATA%/1000# ELSE X(I%)=IDATA%/10#
1620 IF METER$="f" THEN 1640
1630 IC%=INP(830):IF (IC% AND DREADY)=DREADY THEN 1630 'wait until pH changes
1640 NEXT I%
1650 REM
1690 REM ********** linear least squares to determine equilibrium *********
1700 SIGX=0: SIGY=0: SIGXY=0: SIGXX=0: SIGYY=0
1710 FOR I%=1 TO NTIMES%
1720 SIGY=SIGY+X(I%) :SIGX=SIGX+I% :SIGXY=SIGXY+I%*X(I%) :SIGXX=SIGXX+I%*I%
1730 SIGYY=SIGYY+X(I%)*X(I%)
1740 NEXT I%
1750 A=(SIGX*SIGY-NTIMES%*SIGXY)/(SIGX*SIGX-NTIMES%*SIGXX) 'slope
1760 B=(SIGY-A*SIGX)/NTIMES% 'intercept
1770 PH(NUMBER%)=SIGY/NTIMES% 'mean value of pH or mV
1780 XXXX=SIGXX-SIGX*SIGX/NTIMES%
```

```
1790 YYYY=SIGYY-SIGY*SIGY/NTIMES%
1800 XIYY=SIGXY-SIGX*SIGY/NTIMES%
1810 IF YYYY(A*XIYY THEN SE=0' ELSE SE=SQR((YYYY-A*XIYY)/(NTIMES%-2))
1820 SIGSL=SE/SQR(XIXX)                'std.error of slope
1822 ENDTIME=FNTIME(TIME$)             'store time at end of read loop
1824 IF ENDTIME<STARTTIME THEN ENDTIME=ENDTIME+86400' 'in case of midnight
1826 ELAPSEDTIME=ENDTIME-STARTTIME     'time for 1 set of readings
1828 DELAY=DELAY+ELAPSEDTIME           'total time since first reading
1830 SIGSL=SIGSL*NTIMES%*60'/ELAPSEDTIME  'st.err. of slope per min
1840 SLOPE=A*NTIMES%*60'/ELAPSEDTIME   'change in pH (or mV) per minute
1850 SUMSQ=0'
1860 FOR I%=1 TO NTIMES%
1870 SUMSQ=SUMSQ+(X(I%)-PH(NUMBER%))*(X(I%)-PH(NUMBER%))
1880 NEXT I%
1890 SD=SQR(SUMSQ/(NTIMES%-1))   'sd. devn of NTIMES% readings
1900 REM if not stable and still time,go back and get 30 more readings
1910 IF TEST THEN RETURN          'to subroutine 5790
1920 IF ABS(SLOPE)>SDPHMAX-SIGSL AND DELAY<MAXDELAY THEN GOTO 1490 'read the met
er again
1930 IF ABS(SLOPE)<SDPHMAX-SIGSL THEN GOTO 2020
1940 REM remove  outliers due to spikes if SDPHMAX exceeded and DELAY>MAXDELAY
1950 N=0: SUM=0'
1960 FOR I%=1 TO NTIMES%
1970 IF ABS(X(I%)-(B+A*I%)) >4*SE THEN GOTO 1995 'spike if >4*se from best line
1980 SUM=SUM+X(I%)
1990 N=N+1
1992 GOTO 2000
1995 SPIKE=TRUE
2000 NEXT I%
2010 PH(NUMBER%)=SUM/N           'new mean value
2020 REM calculate next titrant addition if not the first or second point
2040 IF NUMBER%=1 THEN GOTO 2080 'do not calculate slope on first point
2050 DELTAPH=PH(NUMBER%)-PH(NUMBER%-1)   'change in ph or mv
2060 DELTAV=VOL(NUMBER%)-VOL(NUMBER%-1)  'change in volume
2070 SLOPE=DELTAPH/DELTAV                'slope
2080 IF INCR$="p" OR INCR$="m" THEN GOTO 2100
2090 NPULSES%=CINT(10000*ASTEP): GOTO 2230   'constant volume increments
2100 IF NUMBER%>2 THEN GOTO 2130
2110 IF NUMBER%=2 THEN TWOINC=ONEINC*ASTEP/DELTAPH    'change 2nd step
2115 IF TWOINC>2*ONEINC THEN ONEINC=2*ONEINC
2120 NPULSES%=CINT(10000*ONEINC): GOTO 2230  'first and second increments
2130 REM this part calculates the next volume increment,based on the algorithm
     in Anal.Chim.Acta (143) p69 (1982)
2132 NUM=NUMBER%
2136 N0=NUM: N1=NUM-1: N2=NUM-2
2140 V1=(VOL(N0)-VOL(N2))/(VOL(N1)-VOL(N2))
2150 E=(PH(N0)-PH(N2))/(PH(N1)-PH(N2))
2160 BB=V1*(1-E)/(E-V1)
2170 AA=-BB*(BB+1)
2180 T1=AA*(PH(N1)-PH(N2))
2190 T2=ASTEP*(BB+V1)
2200 V2=(T1*V1-T2*BB)/(T1+T2)
2210 NPULSES%=CINT(10000*(V2*(VOL(N1)-VOL(N2))+VOL(N2)-VOL(N0)))
2220 IF NPULSES%<1 THEN NPULSES%=1  'minimum of 0.0001 mL
2230 IF (NPULSES% MOD 2)=1 THEN NPULSES%=NPULSES%+1   'only even pulses allowed
2250 REM ************************************************************************
2330 IF NOT SPIKE THEN PRINT "   "; ELSE PRINT "***";
2335 PRINT USING " ###";NUMBER%;
2340 PRINT USING "   ####.####  "; VOL(NUMBER%),PH(NUMBER%),A;
2350 IF NUMBER%>1 THEN PRINT USING " ########.# ";SLOPE; ELSE PRINT SPACE$(12);
2360 PRINT USING "     ####";DELAY
2370 IF HARD$="n" THEN GOTO 2420
2380 IF NOT SPIKE THEN LPRINT "   "; ELSE LPRINT "***";
2385 LPRINT USING " ###";NUMBER%;
2390 LPRINT USING "   ####.####  ";VOL(NUMBER%),PH(NUMBER%),A;
2400 IF NUMBER%>1 THEN LPRINT USING " ########.# ";SLOPE;ELSE LPRINT SPACE$(12);
```

```
2410 LPRINT USING "     ####";DELAY
2420 REM terminate the titration if necessary
2430 REM comparison of the first reading and ENDPH determines trend of curve
2440 IF PH(1)<ENDPH AND PH(NUMBER%)>ENDPH THEN GOSUB 2540
2450 IF PH(1)>ENDPH AND PH(NUMBER%)<ENDPH THEN GOSUB 2540
2460 REM
2470 VOL(NUMBER%+1)=VOL(NUMBER%)+NPULSES%/10000
2471 IF VOL(NUMBER%+1)<= ENDVOL THEN GOTO 2490
2472 ENDVOL=VOL(NUMBER%+1)+1!
2473 COLOR 31,0          'flash the refill message
2474 PRINT"          --- The buret needs to be refilled"
2475 GOSUB 2950
2476 COLOR 7,0: LOCATE CSRLIN-1,POS(0)    'overwrite the refill message
2477 REM
2490 NUMBER%=NUMBER%+1
2500 GOSUB 3990      'add the titrant
2510 GOTO 1460
2520 REM ****************** titration termination ***************************
2530 REM
2540 COL(DPAGE%)=POS(1):ROW(DPAGE%)=CSRLIN  'store current cursor position
2550 LOCATE 25,1:PRINT SPACE$(79);:LOCATE 25,1
2560 PRINT "Do you REALLY want to stop the titration? (y/n)";
2570 ANS$=INKEY$ :IF ANS$="" THEN 2570
2580 ANS$=MID$(ANS$,1,1)
2590 IF (ANS$="y" OR ANS$="Y") THEN GOTO 2680 ELSE GOSUB 3130
2600 LOCATE 25,1                  'print the key information
2610 COLOR 0,7:PRINT " F1 ";:COLOR 7,0:PRINT " Pause   ";
2620 COLOR 0,7:PRINT " F2 ";:COLOR 7,0:PRINT " Parameter Revision ";
2630 COLOR 0,7:PRINT " F3 ";:COLOR 7,0:PRINT " Stop Titration ";
2640 COLOR 0,7:PRINT " F4 ";:COLOR 7,0:PRINT " Screen ";
2650 COLOR 0,7:PRINT " F5 ";:COLOR 7,0:PRINT " Test ";
2660 LOCATE ROW(DPAGE%),COL(DPAGE%)
2670 RETURN
2680 LOCATE ROW(DPAGE%),COL(DPAGE%)
2690 FOR II=1 TO NUMBER%-2
2700 IF DPANS$="M" THEN PRINT#2, "     ";
2710 PRINT#2, TAB(ITAB);
2720 PRINT#2, USING " ####.####"; VOL(II),PH(II)
2730 NEXT II
2740 IF DPANS$="M" THEN PRINT#2, "   -1";
2750 PRINT#2, TAB(ITAB);
2760 PRINT#2, USING " ####.####"; VOL(NUMBER%-1),PH(NUMBER%-1)
2770 PRINT#2," "
2780 PRINT:PRINT "Titration terminated at ";TIME$
2790 IF HARD$="n" THEN GOTO 2810
2800 LPRINT:LPRINT "Titration terminated at ";TIME$
2810 KEY OFF
2820 PRINT
2830 PRINT "The titration data is stored in ";DATFILE$;" using parameters in ";P
ARFILE$
2840 PRINT: PRINT "Press F2 or type RUN to restart....."
2850 OUT &20,4
2860 OUT &21,128
2870 CLOSE #1
2880 CLOSE #2
2890 IF HARD$="n" THEN GOTO 2910
2900 LPRINT "The titration data is stored in ";DATFILE$;" using parameters in ";
PARFILE$
2910 POKE &H5B,1    'return top of scroll screen to line 1
2920 ON ERROR GOTO 0      'disable error trapping
2930 END
2940 REM
2950 REM ******* subroutine to temporarily halt the titration ****************
2960 COL=POS(N):ROW=CSRLIN    'store current cursor position in active page
2970 LOCATE 25,1:PRINT SPACE$(79);:LOCATE 25,1
2980 COLOR 0,7:PRINT " F1 ";:COLOR 31,0:PRINT " Pause   ";
2990 COLOR 7,0:PRINT "Press the space bar to resume the titration ...";
3000 IF INKEY$<>CHR$(32) THEN 3000
3010 LOCATE 25,1
```

```
3020 COLOR 0,7:PRINT " F1 ";:COLOR 7,0:PRINT " Pause   ";
3030 COLOR 0,7:PRINT " F2 ";:COLOR 7,0:PRINT " Parameter Revision ";
3040 COLOR 0,7:PRINT " F3 ";:COLOR 7,0:PRINT " Stop Titration ";
3050 COLOR 0,7:PRINT " F4 ";:COLOR 7,0:PRINT " Screen ";
3060 COLOR 0,7:PRINT " F5 ";:COLOR 7,0:PRINT " Test ";
3070 LOCATE ROW,COL
3080 RETURN
3090 END
3100 REM
3110 REM *************** subroutine for parameter revision ********************
3120 COL(DPAGE%)=POS(0):ROW(DPAGE%)=CSRLIN    'store current cursor position
3130 SCREEN 0,0,PPAGE%,PPAGE% :VPAGE%=PPAGE%:APAGE%=PPAGE%
3140 LOCATE 25,1
3150 COLOR 0,7:PRINT " F1 ";:COLOR 7,0:PRINT " Pause   ";
3160 COLOR 0,7:PRINT " F2 ";:COLOR 31,0:PRINT " Parameter Revision ";
3170 COLOR 0,7:PRINT " F3 ";:COLOR 7,0:PRINT " Stop Titration ";
3180 COLOR 0,7:PRINT " F4 ";:COLOR 7,0:PRINT " Screen ";
3190 COLOR 0,7:PRINT " F5 ";:COLOR 7,0:PRINT " Test ";
3200 LOCATE ROW(PPAGE%),COL(PPAGE%)
3210 REVISE=TRUE
3220 GOTO 3650
3230 GOSUB 2950
3240 REVISE=FALSE
3250 SCREEN 0,0,DPAGE%,DPAGE%:VPAGE%=DPAGE%:APAGE%=DPAGE%
3260 LOCATE ROW(DPAGE%),COL(DPAGE%)   'go to previous position on data page
3270 RETURN
3280 END
3290 REM
3300 REM
3310 REM ****************** parameter input subroutine ***************************
3320 PRINT:INPUT "Are the titration PARAMETERS already stored in a disk file? (y
/n)";PAR$
3330 PAR$=MID$(PAR$,1,1)
3340 IF PAR$="N" THEN PAR$="n"
3350 IF PAR$="Y" THEN PAR$="y"
3360 IF PAR$="y" OR PAR$="n" THEN GOTO 3380 ELSE LOCATE CSRLIN-2,POS(0)
3370 SOUND 300,5 :GOTO 3320
3380 IF PAR$="y" THEN INPUT "Enter the name of this file:",PARFILE$      ELSE GO
TO 3580
3390 PARFILE$=DISK$+PARFILE$
3400 OPEN PARFILE$ FOR INPUT AS #1
3410 INPUT#1,INCR$,ENDPH,ENDVOL,ONEINC,ASTEP,MAXDELAY,SDPHMAX,MODE$
3440 PRINT:INPUT "Enter a short, descriptive title IN CAPITALS:";TITLE$:PRINT
3450 IF HARD$="y" THEN LPRINT "      ";TITLE$
3460 PRINT "The following titration parameters will be used:":PRINT
3470 IF MODE$="m" THEN PRINT "end mV ";ENDPH ELSE PRINT "end pH ";ENDPH
3480 PRINT "maximum volume of titrant";ENDVOL;" mL."
3490 IF INCR$="m" THEN PRINT "mV increment ";ASTEP:GOTO 3510
3500 IF INCR$="p" THEN PRINT "pH increment ";ASTEP ELSE PRINT "volume increment
";ASTEP
3510 IF INCR$="v" THEN GOTO 3520 ELSE PRINT "the first two volume additions will
 be ";ONEINC;"mL each."
3520 PRINT "maximum equilibration delay ";MAXDELAY;" seconds"
3530 PRINT "maximum allowed change of ";
3540 IF MODE$="p" THEN PRINT "pH"; ELSE PRINT "mV";
3550 PRINT " per min. for equilibrium ";SDPHMAX
3560 GOTO 3900
3570 REM **** entry to parameter revision if no file already exists *******
3580 PRINT:PRINT "then enter the parameters now.....":PRINT
3590 PRINT:INPUT "Enter a short, descriptive title IN CAPITALS:";TITLE$:PRINT
3600 IF HARD$="y" THEN LPRINT "      ";TITLE$
3610 INPUT "Will the meter readings be in mV or pH (mV/pH)";MODE$
3620 MODE$=MID$(MODE$,1,1)
3622 IF MODE$="M" THEN MODE$="m"
3624 IF MODE$="P" THEN MODE$="p"
3630 IF MODE$="m" OR MODE$="p" THEN 3650 ELSE LOCATE CSRLIN-1,POS(0)
```

```
3640 SOUND 300,5:GOTO 3610
3650 IF MODE$="m" THEN INPUT "end mV ";ENDPH ELSE INPUT "end pH ";ENDPH
3660 IF MODE$="m" THEN INPUT "Will this titration be in constant mV or volume in
crements (mV/vol)";INCR$ ELSE GOTO 3700
3670 INCR$=MID$(INCR$,1,1)
3672 IF INCR$="M" THEN INCR$="m"
3674 IF INCR$="V" THEN INCR$="v"
3680 IF INCR$="m" OR INCR$="v" THEN 3740 ELSE LOCATE CSRLIN-1,POS(0)
3690 SOUND 300,5: GOTO 3660
3700 IF MODE$="p" THEN INPUT "Will this titration be in constant pH or volume in
crements ";INCR$
3710 INCR$=MID$(INCR$,1,1)
3712 IF INCR$="P" THEN INCR$="p"
3714 IF INCR$="V" THEN INCR$="v"
3720 IF INCR$="p" OR INCR$="v" THEN 3750 ELSE LOCATE CSRLIN-1,POS(0)
3730 SOUND 300,5: GOTO 3700
3740 IF INCR$="m" THEN INPUT "mV increment ";ASTEP:GOTO 3760
3750 IF INCR$="p" THEN INPUT "pH increment ";ASTEP ELSE INPUT "volume increment
";ASTEP
3760 IF INCR$<>"v" AND NUMBER%=1 THEN INPUT "What do you want the first two volu
me additions to be";ONEINC
3770 INPUT "maximum equilibration delay (seconds) ";MAXDELAY
3780 PRINT "maximum allowed change of ";
3790 IF MODE$="p" THEN PRINT "pH"; ELSE PRINT "mV";
3800 INPUT " per min. for equilibrium ";SDPHMAX
3810 PRINT
3820 ROW(PPAGE%)=CSRLIN: COL(PPAGE%)=POS(0) 'store postion on parameter page
3830 IF REVISE=TRUE THEN GOTO 3230
3840 INPUT "What is the file in which these PARAMETERS will be stored";PARFILE$
3850 IF PARFILE$<>"" THEN GOTO 3870 ELSE LOCATE CSRLIN-1,POS(0)
3860 SOUND 300,5:GOTO 3840
3870 PARFILE$=DISK$+PARFILE$
3880 OPEN PARFILE$ FOR OUTPUT AS #1
3890 PRINT#1,INCR$;",";ENDPH;ENDVOL;ONEINC;ASTEP;MAXDELAY,SDPHMAX,MODE$
3900 PRINT:INPUT"What is the file in which the DATA is to be stored";DATFILE$
3910 IF DATFILE$<>"" THEN GOTO 3930 ELSE LOCATE CSRLIN-2,POS(0)
3920 SOUND 300,5: GOTO 3900
3930 DATFILE$=DISK$+DATFILE$
3940 OPEN DATFILE$ FOR OUTPUT AS #2
3950 RETURN
3960 END
3970 REM
3980 REM ************************************************************************
3990 REM subroutine to put out a train of TTL pulses from [p5] pin 10
       (9513 timer OUT5) every 20msec
4000 REM
4010 KEY(1) STOP:KEY(2) STOP:KEY(3) STOP:KEY(4) STOP:KEY(5) STOP 'disable keys
4020 OUT 825,208          'disarm counter 5                              1101 0000
4030 OUT 825,23           'data pointer set to master mode register      0001 0111
4040 OUT 824,0            'low byte                                      0000 0000
4050                      'FOUT source=F1
4060                      'comparators disabled
4070                      'time-of-day disabled
4080 OUT 824,192          'high byte                                     1100 0000
4090                      'scalar control in BCD
4100                      'enable increment
4110                      '8 bit bus
4120                      'FOUT on
4130                      'FOUT divide by 1
4140 FOR I%=1 TO NPULSES%
4150 OUT 825,5            'set data pointer to counter 5 mode register 0000 0101
4160 OUT 824,84           'low byte                                      0101 0100
4170                      'disable special gate
4180                      'reload from load or hold
4190                      'count down once in BCD
4200 OUT 824,15           'high byte                                     0000 1111
4210 REM use of F5 sets smallest time interval of 0.01sec
4220 OUT 824,10           'low byte of counter 5 load register
4230 OUT 824,0            'high byte
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4240                    'no gating
4250                    'count on rising edge of F5
4260 OUT 825,112        'load and arm counter 5                         0111 0000
4270 OUT 825,237        'set OUT5 high                                  1110 0101
4280 NEXT I%
4290 OUT 825,229        'sets OUT5 low                                  1110 1101
4300 KEY(1) ON:KEY(2) ON:KEY(3) ON:KEY(4) ON:KEY(5) ON  're-enable keys
4310 RETURN
4320 END
4330 REM ***************** error trapping subroutine ***************************
4340 SOUND 300,5
4350 IF ERR=27 THEN PRINT "The printer is out of paper.":GOSUB 2950:RESUME
4360 IF ERR=50 THEN PRINT "Field overflow":RESUME 2490
4370 IF ERR=24 THEN PRINT "A device, probably the printer, has shut off -- turn
it back on.":GOSUB 2950:RESUME
4380 IF ERR=53 THEN PRINT "File ";PARFILE$;" not found ":FILES DISK$+"*.*" :RESU
ME 3380
4390 IF ERR=52 AND ERL=3940 THEN PRINT "This file name is invalid...use a differ
ent file name" :RESUME 3900 'checks for
4400 IF ERR=52 AND ERL=3880 THEN PRINT "This file name is invalid...use a differ
ent file name" :RESUME 3840 'checks for
4410 IF ERR=70 THEN PRINT "Disk ";DISK$;" is write protected - use another or re
move the write-protect tab":GOSUB 2950: RESUME 3900
4420 IF ERR=71 THEN PRINT "Put the disk in !!":GOSUB 2950:RESUME 3840
4430 POKE &H5B,1
4440 ON ERROR GOTO 0
4450 END
4460 REM *************data input format for MINIQUAD81 ********************
4470 LARS=1 'every point is used
4480 INPUT "How many formation constants are there in this system ";NK
4490 INPUT "How many of these will be determined with MINIQUAD81 ";N
4500 MAXIT=50 'maximum number of iterations
4510 IPRIN=0 'iterations are not monitored
4520 PRINT:INPUT "What is the number of reactants (mass balance equations=#ligan
ds+#metals+1) ";NMBE
4530 NC=NMBE-1 'number of unknown free concs. per data point
4540 PHCAL=1!
4550 IF MODE$="m" THEN INPUT "Do you want to refine Eo  (Yes/No) ";ANS$
4560 IF MODE$="p" THEN INPUT "Do you want to refine pHcal (Yes/No) ";ANS$
4570 ANS$=MID$(ANS$,1,1)
4580 IF ANS$="Y" OR ANS$="y" THEN IREF=1 ELSE IREF=0
4590 TEMP=25! 'degrees C.
4600 PRINT#1,LARS,NK,N,MAXIT,IPRIN,NMBE,NC,PHCAL,IREF,TEMP
4610 PRINT:PRINT:PRINT "Now start entering the LOGS of the formation constants,
or estimations of such:"
4620 FOR III=1 TO NK
4630 PRINT:PRINT:PRINT "Constant number ";III;".....";:INPUT;XL6CONST
4640 JPOT(III)=INT(XL6CONST)
4650 BETA(III)=10!^(XL6CONST-JPOT(III))
4660 PRINT "What are the reactant stoichiometric coeffs at this pK, in the order
 of metal (if present),ligand(s),then protons...
4670 PRINT#1,JPOT(III),BETA(III)
4680 FOR JJJ=1 TO NMBE
4690 PRINT "              --- of reactant ";JJJ;
4700 INPUT JQR1(III,JJJ)
4710 PRINT#1,JQR1(III,JJJ)
4720 NEXT JJJ
4730 INPUT "Will this formation constant be refined  (Yes/No) ";ANS$
4740 ANS$=MID$(ANS$,1,1)
4750 IF ANS$="Y" OR ANS$="y" THEN IKEY(III)=1 ELSE IKEY(III)=0
4760 PRINT#1,IKEY(III)
4770 NEXT III
4780 IF MODE$="m" THEN JEL=1 ELSE JEL=0
4790 PRINT#1,JEL
4800 PRINT:PRINT "Now enter in the initial number of moles each reactant (negat
ive if OH-) in the TITRATE:"
4810 REM
4820 FOR IKK=1 TO NMBE
4830 PRINT "              --- moles reactant ";IKK;
4840 INPUT TOTC(IKK)
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4850 PRINT#1,TOTC(IKK)
4860 NEXT IKK
4870 IF JEL=1 THEN INPUT "What is the Eo value ";E0 ELSE E0=0!
4880 PRINT#1,E0
4890 PRINT "What are the concentrations of each reactant in the TITRANT (negativ
e if OH- ) :"
4900 FOR LLL=1 TO NMBE
4910 PRINT "              --- of reactant ";LLL;
4920 INPUT ADDC1(LLL)
4930 PRINT#1,ADDC1(LLL)
4940 NEXT LLL
4950 INPUT "What is the initial volume of the solution (mL)";VINIT
4960 PRINT#1,VINIT
4970 PRINT:PRINT:PRINT:PRINT "WASN'T THAT EASY?!":PRINT:PRINT
4980 REM formatting the initial data for miniquad
4990 REM
5000 FOR I=1 TO 10:PRINT#2,:NEXT I
5010 PRINT#2," ";TITLE$,DATE$
5020 PRINT#2, USING "   ##";LARS,NK,N,MAXIT,IPRIN,NMBE,NC
5030 PRINT#2, USING "#.###";PHCAL;
5040 PRINT#2, USING" #";IREF;
5050 PRINT#2,"  0"
5060 PRINT#2, USING "    ####.#";TEMP
5070 FOR NNN=1 TO NK
5080 PRINT#2, USING "  ##.#####";BETA(NNN);
5090 PRINT#2, USING "  ###";JPOT(NNN);
5100 FOR MMM=1 TO NMBE
5110 PRINT#2,USING "  ###";JQR1(NNN,MMM);
5120 NEXT MMM
5130 PRINT#2,USING "  ###";IKEY(NNN)
5140 NEXT NNN
5150 PRINT#2, USING"    #";JEL
5160 FOR III=1 TO NMBE
5170 PRINT#2, USING "####.#####";TOTC(III);
5180 NEXT III
5190 PRINT#2,"     "
5200 PRINT#2, USING "  ####.###";E0
5210 FOR JJJ=1 TO NMBE
5220 PRINT#2, USING " ####.####";ADDC1(JJJ);
5230 NEXT JJJ
5240 PRINT#2,USING " #####.###";VINIT
5250 RETURN 1090
5260 REM subroutine to get miniquad parameters from parfile$
5270 INPUT#1,LARS,NK,N,MAXIT,IPRIN,NMBE,NC,PHCAL,IREF,TEMP
5280 PRINT:PRINT "There are ";NK;" formation constants, ";N;" to be refined..."
5290 PRINT:PRINT "  log(beta)                refined ?"
5300 FOR I=1 TO NK
5310 INPUT#1,JPOT(I),BETA(I)
5320 PRINT USING "##.#### _E";BETA(I);:PRINT USING "+##      ";JPOT(I),
5330 FOR J=1 TO NMBE
5340 INPUT#1,JQR1(I,J)
5350 PRINT JQR1(I,J);
5360 NEXT J
5370 INPUT#1,IKEY(I)
5380 IF IKEY(I)=1 THEN PRINT ,"Yes" ELSE PRINT ,"No"
5390 NEXT I
5400 INPUT#1, JEL
5410 GOTO  4800
5420 GOSUB 4990
5430 RETURN
5440 END
5450 REM *************** subroutine to flip pages 0 and 1 ******************
5460 IF VPAGE%=DPAGE% THEN SCREEN 0,0,APAGE%,0 ELSE SCREEN 0,0,APAGE%,1
5470 IF VPAGE%=1 THEN VPAGE%=0 ELSE VPAGE%=1
5480 RETURN
5490 END
5500 REM **********subroutine to continuously read the pH meter ***************
5510 TEST=TRUE            'set the test flag
5520 KEY(1) OFF:KEY(2) OFF 'temporarily turn keys off
5530 KEY(3) OFF:KEY(4) OFF:KEY(5) OFF
5540 ON KEY(6) GOSUB 5850:KEY(6) STOP 'program key F6 to get out of this
```

```
5550 COL(DPAGE%)=POS(0):ROW(DPAGE%)=CSRLIN 'store cursor position on data page
5560 SCREEN 0,0,TPAGE%,TPAGE%  'flip to test page 2
5570 CLS
5580 LOCATE 25,1        'set up line 25
5590 COLOR 23,0:PRINT " Meter Test    ";:COLOR 7,0
5600 PRINT"   press ";:COLOR 0,7:PRINT " F6 ";:COLOR 7,0:
5610 PRINT" to return"
5620 LOCATE 1,1
5630 PRINT "        "+CHR$(179)
5640 FOR I=1 TO 10:PRINT "        "+CHR$(195):NEXT I
5650 PRINT "        "+CHR$(195)+STRING$(70,196)
5660 FOR I=1 TO 10:PRINT "        "+CHR$(195):NEXT I
5670 PRINT "        "+CHR$(179)
5680 KEY(6) ON
5690 GOSUB 1460         'go and read the meter
5700 KEY(6) OFF
5710 LOCATE 12,1        'start printing ph meter readings
5720 IF MODE$="p" THEN PRINT USING "##.####";PH(NUMBER%)
5730 IF MODE$="m" THEN PRINT USING "####.##";PH(NUMBER%)
5740 FOR I%=1 TO NTIMES%
5750 IF METER$="o" THEN ICOL=I% ELSE ICOL=1+I%\3
5760 IF MODE$="p" THEN IROW=12-(X(I%)-PH(NUMBER%))/.001 ELSE IROW=12-(X(I%)-PH(N
UMBER%))/.1
5770 IF IROW<1 THEN IROW=1
5780 IF IROW>23 THEN IROW=23
5790 LOCATE IROW,2+ICOL+9 :PRINT CHR$(254)
5800 NEXT I%
5810 LOCATE 25,40:PRINT USING " ##.####";SLOPE;
5820 PRINT "(";:PRINT USING " ##.####";SIGSL;:PRINT ")"
5830 FOR I=1 TO 1000:NEXT I
5840 GOTO 5570          'read the meter continuously unless F6 is pressed
5850 REM return from test routine
5860 SCREEN 0,0,TPAGE%,DPAGE%:  'flip back to data page
5870 KEY(6) ON
5880 TEST=FALSE          'reset the test flag
5890 SCREEN 0,0,DPAGE%,DPAGE%
5900 LOCATE 25,1         'set up old line 25 key again
5910 COLOR 0,7:PRINT " F1 ";:COLOR 7,0:PRINT " Pause   ";
5920 COLOR 0,7:PRINT " F2 ";:COLOR 7,0:PRINT " Parameter Revision ";
5930 COLOR 0,7:PRINT " F3 ";:COLOR 7,0:PRINT " Stop Titration ";
5940 COLOR 0,7:PRINT " F4 ";:COLOR 7,0:PRINT " Screen ";
5950 COLOR 0,7:PRINT " F5 ";:COLOR 7,0:PRINT " Test ";
5960 LOCATE ROW(DPAGE%),COL(DPAGE%)   'go to previous position on data page
5970 KEY(1) ON:KEY(2) ON:KEY(3) ON:KEY(4) ON  'turn keys on again
5980 ON KEY(5) GOSUB 5500:KEY(5) ON
5990 RETURN
```

Sample Parameter Set-ups

1. New set of titration parameters

```
          IBM PC Titration Program          07-11-1983  12:39:37

Are the titration parameters already stored in a disk file (y/n)? no
then enter the parameters now.....

Enter a short, descriptive title IN CAPITALS:  TITRATION  1

Will this the meter readings be in mV or pH (mV/pH)? mv
end mV? -400
Will this titration be in constant mV or volume increments(mV/pH)?mv
mV increment?  -10
What do you want the first two volume additions to be? 0.2
maximum equilibration delay (seconds)?  120
maximum allowed change of mV per min. for equilibrium ? 0.1

What is the file in which these PARAMETERS will be stored?mvmv10.par

What is the file in which the DATA is to be stored? jul11001.dat
Will this data be processed with MINIQUAD81, or in another way (m/o)? o
What is the initial burette volume (usually 0,of course)? 1.2345

****  MAKE SURE THAT THE 1.000mL BURETTE IS READY  *****
```

2. Using parameters already stored

```
Are the titration PARAMETERS already stored in a disk file (y/n)? y
Enter the name of this file: mvmv10.par

Enter a short, descriptive title IN CAPITALS:  TITRATION Δ2

The following titration parameters will be used:

end mV -400
maximum volume of titrant 1 mL.
mV increment -10
the first two volume additions will be 0.2 mL each
maximum equilibration delay 120 seconds
maximum allowed change of mV per min. for equilibrium. 0.1

What is the file in which the DATA is to be stored? jul11002.dat
Will this data be processed with MINIQUAD81, or in another way (m/o)? o
What is the initial burette volume (usually 0, of course)? 0.0000

*****  MAKE SURE THAT THE 1.000ML BURETTE IS READY  *****
```

If MINIQUAD81 is to be used for Data Processing:

MINIQUAD81 calculates the overall association constants called β's, according to the following relationships:

$$pM + qL + rH \longleftrightarrow M_pL_qH_r$$

$$\beta_{pqr} = \frac{[M_pL_qH_r]}{[M]^p[L]^q[H]^r}$$

where M, L and H represent metal, ligand and protons, respectively with the stiochiometric coefficients p, q and r.

The program will format the data for direct use with MINIQUAD81 (MINIQ81DOPTH) if specified. What follows is a listing of the prompting for setting the initial program parameters - how many constants are to be determined, initial mmoles reactants, etc. - with sample responses included.

```
Will this data be processed with MINIQUAD81, or in another way? m
```

If these parameters have already been stored in the parameter file opened for this titration they will be written to the terminal screen for you to see. Otherwise, you will enter them in as follows:

```
How many formation constants are there in this system ? 2
How many of these will be determined with MINIQUAD81 ?  1


What is the number of reactants (mass balance equations=aligands+
  ∆metals+1)?  3


Do you want to refine Eo  (Yes/No)?  yes          - or PHCAL if
                                                     pH mode


Now start entering the LOGS of the formation constants, or
estimations of such:

Constant number  1 .....   10.745
What are the reactant stoichiometric coefficients at this logK,
in the order of metal (if present), ligand(s), then protons (if
OH-, enter a negative coeff.)....?
            --- of reactant  1?  1        eg. for MLH2 complex
            --- of reactant  2?  1             then 1 1 2:
            --- of reactant  3?  2
Will this formation constant be refined  (Yes/No)?  no
```

Prompting for entering formation constants will continue until the number of formation constants for the experimental system specified above is reached (here, 2).

```
Now enter the initial number of mmoles or each reactant (negative
if OH-) in the TITRATE:
            --- mmoles reactant 1?  0.4573
            --- mmoles reactant 2?  0.479089
            --- mmoles reactant 3?  1.45384
What is the Eo value? 440.5          - not asked if using pH
What are the concentrations of each reactant in the TITRANT
(negative if OH-) :
            --- of reactant 1?  0
            --- of reactant 2?  0
            --- of reactant 3?  -1.0034
What is the initial volume of the solution(mL)? 24.506
```